FINAL REPORT

Development of Toxicity Data for Munition Compounds to Support Toxicity Reference Value Derivations for Wildlife

SERDP Project ER-1420
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EXECUTIVE SUMMARY
FINAL REPORT
TOXICOLOGY REPORT NO. 87-XE-06ED-05
DEVELOPMENT OF TOXICITY DATA FOR MUNITIONS COMPOUNDS TO SUPPORT TOXICITY REFERENCE VALUE DERIVATION FOR WILDLIFE
JANUARY 2005–JANUARY 2010

1. PURPOSE. To develop toxicity data for energetic compounds in representative wildlife models to be used in toxicity reference value (TRV) derivation. These values can then be used to derive soil screening levels for these military-specific chemicals.

2. BACKGROUND. Military activities associated with training, munitions manufacturing, and demilitarization have resulted in soil, surface water, and sediment contamination with munitions compounds and their breakdown products. Since these areas of contamination often include wildlife habitat, risk incurred from exposure needs to be evaluated. This is particularly important in a risk management context when balancing the potential for adverse effects from exposure with habitat alterations associated with cleanup operations. To determine safe levels of exposure, toxicity data from controlled laboratory studies are needed. This information can then be used to develop TRVs for mammals, birds, reptiles, and terrestrial amphibians.

3. METHODS.

a. Over the course of 4 years, studies were conducted to evaluate the effects of various munitions in representative bird, mammalian, reptile, and amphibian species that could be exposed to these compounds from soil releases. Specific studies were designed to consider primary environmental exposure routes, ecologically important health effects, application of toxicological data in a risk assessment context, and a reduction in the uncertainty in the derivation of thresholds for adverse effects (i.e., TRV derivation). Selection of compounds was based on a lack of class-specific information and reported prevalence in the environment at military installations. Work was leveraged from other programs where toxicological studies were already underway.

b. Wildlife models used for the various animal classes included Western fence lizards (*Sceloporus occidentalis*; reptiles), red-backed salamander (*Plethodon cinereus*; amphibians), Northern bobwhite (*Colinus virginanus*; birds), and the white-footed mouse (*Peromyscus leucopus*; mammals). Compounds tested included:2,4,6-trinitrotoluene (or TNT); 2,4- and 2,6-dinitrotoluene (or DNT); 2-amino-4,6-dinitrotoluene

and/or 4-amino-2,6-dinitrotoluene (A-DNT); octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (or HMX); and 1,3,5-trinitrohexahydro-1,3,5-triazine (or RDX). Each study was performed according to an approved protocol with the results published in the peer-reviewed literature. Currently, there are two studies still in preparation. These data have and will be integrated into the chemical-specific Wildlife Toxicity Assessment profiles and used to derive TRVs for site-specific risk assessment purposes.

4. CONCLUSIONS. Toxicity of tested energetics and associated breakdown products resulted in varying effects relative to the laboratory species tested. Differences in effects were largely due to physiological differences between vertebrate class representatives and are believed to be useful laboratory models for future toxicology research. These data have provided valuable insight into ecotoxicological risk assessment and serve as a valuable surrogate for estimating effects to species in field situations where questions regarding the relative risk of exposure to energetic compounds exist.

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JANUARY 2005–JANUARY 2010

1. REFERENCES. See Appendix A for a listing of references.

2. PURPOSE. To develop toxicity data in representative wildlife models for energetic compounds to be used in toxicity reference value (TRV) derivation. These values can then be used to derive soil screening levels for these military-specific chemicals.

3. AUTHORITY. To ensure environmental, safety, and occupational health as part of the responsibilities outlined in Army Regulation 200-1 (Environmental Protection and Enhancement), this research was completed as part of the U.S. Army Public Health Command (Provisional)'s (USAPHC (Prov), formerly known as the U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM)) responsibility to ensure environmental health in support of military readiness.

4. GENERAL BACKGROUND.

a. National Defense requires that the Armed Forces be in a ready, trained state at all times. Maintaining training activities at U.S. Army installations is crucial for the military to be operational and function at the desired level of performance. Military personnel involved in training exercises may be exposed to substances that may compromise their health. Moreover, land used as part of training activities must also support vital natural resources and wildlife habitat, and therefore must be used in an environmentally sustainable manner.

b. Military activities associated with training, munitions manufacturing, and demilitarization have resulted in soil, surface water, and sediment contamination with munition compounds and their breakdown products. Since these areas of contamination often include wildlife habitat, risk incurred from exposure needs to be evaluated. This is particularly important in a risk management context when balancing the potential for adverse effects from exposure against habitat alterations associated with cleanup operations. To determine safe levels of exposure, toxicity data from controlled laboratory studies are needed. Only data from controlled studies can be used to derive safe levels of exposure for valued resources at sites worldwide. This information is then used to develop TRVs for mammals, birds, reptiles, and terrestrial amphibians.

c. This task is focused on military-unique compounds, including the energetics and explosive compounds lacking toxicological data for specific terrestrial vertebrate classes. All studies followed a progression of data collection that began with acute exposures, then repetitive subacute exposures, and ending with subchronic (>10 percent of lifespan) exposures. With the exception of the salamander exposures, all compounds were administered via gavage using measured daily doses specific to individual body mass and treatment. Since amphibians are sensitive to dermal exposures, particularly for energetics, and because of difficulties involved in the oral administration of compounds, salamanders were exposed to compounds through the soil matrix. Another exception was the subchronic exposure of 2-amino-4,6-dinitrotoluene and/or 4-amino-2,6-dinitrotoluene (A-DNT) to *P. leucopus* via their feed; this was necessary given the excessive stress incurred from oral-dosing procedures experienced during the subacute test.

5. OBJECTIVE.

a. Since wildlife species can have large home ranges and occupy various habitats, exposures can vary. It is, therefore, important that the Department of Defense has controlled laboratory toxicity data for wildlife species that can be used at any installation. In this way, site-specific concerns can be addressed through site-specific exposure adjustments and then compared with laboratory toxicity data to determine the potential for adverse effects.

b. The objective of this work was to fill the primary toxicological data gaps for a representative laboratory animal model for each vertebrate class (i.e., mammals, birds, reptiles, and amphibians) from where toxicity data are lacking. Each series of tests followed a logical time/concentration paradigm from acute (lethal), subacute, to subchronic (sublethal) testing as mentioned. Exceptions included a terrestrial amphibian test where a 28-day subchronic soil exposure study followed a 10-day range-finding test. Each study was conducted according to an approved protocol by the Institutional Animal Care and Use Committee (IACUC) and conducted in our AAALAC-accredited animal care facility at USAPHC (Prov). Each study was conducted consistent with Good Laboratory Practices (GLPs)—the same standard used for nonclinical human toxicology testing ensuring the highest data quality.

c. Although reports were generated from the results of these assays, acceptability of toxicological information for use in a risk assessment context is increased when data are published within scientific journals and peer-reviewed manuscripts. Therefore, the following report contains much of the information published in those journals and manuscripts. See Appendix B for a listing of all peer-reviewed manuscripts published or under review to date.

6. METHODS.

a. Reptile, Avian, and Mammalian Toxicology.

(1) Test Animal and Husbandry. Mature laboratory-reared Western fence lizards were obtained from Oklahoma State University; rabbits were obtained from Charles River Laboratories (Wilmington, Massachusetts, USA); Northern bobwhites were obtained from Trace Pheasantry (Douglassville, Pennsylvania, USA); and white-footed mice were obtained from University of South Carolina. All animals were housed individually either in modified polycarbonate mouse cages or wire bottom stainless steel cages (birds and rabbits), assigned a unique identification number, and acclimated for 2 weeks while being observed for any abnormal behavior or possible ill health. Food included live crickets for lizards, quail feed for birds, rabbit chow for the rabbits, and mouse feed for the mice. The study room temperatures ranged from 21 degrees Celsius (°C) to 29 °C. For lizards, a 7–9 °C temperature gradient was established using Flex Watt heat tape (6 W/ft) placed under each row of the cages so that the temperature at the rear of the cage reached approximately 32 °C. Mammals were maintained on a 12 light (12L):12 dark (12D) photoperiod, lizards on a 14 hour light (L):10 hour dark (D), and birds on a 12L:8D photoperiod. Relative humidity ranged between 30 percent and 70 percent. Polycarbonate-bottom cages contained woodchip bedding (P.J. Murphy Forest Products, Montville, New Jersey, USA). Various forms of environmental enrichment were used (lizards: 10-centimeter polyvinyl chloride tube, birds: sisal rope, rabbits: play ball, and mice: timothy cubes). Daily observations were made on condition (lethargy or morbidity) as well as post-dose behavioral observations. Animals were considered moribund and euthanized for humane considerations when they lost a significant amount of body mass (> 20-30 percent), did not respond to stimulus, and/or lacked righting reflex. Anesthesia prior to euthanasia was achieved via carbon dioxide exposure, and euthanasia was completed through decapitation and/or exsanguination (lizards, birds), isoflurane then exsanguination (mice), or intravenous administration of a ketamine/xylazine cocktail followed by an injection of pentabarbital (rabbits).

(2) Chemicals. Test compounds were obtained from a variety of sources (e.g., 2,6-dinitrotoluene (DNT) was obtained from Sigma-Aldrich (Sigma-Aldrich Corporation St Louis, Missouri, USA); 2,4,6-trinitrotoluene (TNT); 1,3,5-trinitrohexahydro-1,3,5-triazine (RDX); and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) from Naval Surface Warfare Center, Indian Head Division (Indian Head, Maryland, USA); and A-DNT from Ministry of National Defense (Valcartier, Quebec, Canada)). Test compound purity met or exceeded 97 percent. Test compounds were dissolved in either methylcellulose (MC) (DNT; MC, Fisher Scientific Suwanee, Georgia, USA) with subsequent deionized water dilution, or into corn oil as a vehicle. Concentrations of dosing solutions were confirmed using a modified U.S. Environmental Protection

Agency (EPA) Method 8330 as described (reference 1). For analysis by gas chromatography, dosing solutions were diluted with isoamyl acetate,then analyzed on a gas chromatograph using a [identify column type] column. The gas chromatograph was fitted with an electron capture detector. All dosing solution concentrations were verified and determined to be stable for 60 days prior to the onset of the study.

(3) Dosing Methods. Lizards, quail, and mice were orally dosed via a species-appropriate method. For lizards, doses were administered using a calibrated Fisherbrand® Finnpipette® II pipette to the rear of the mouth, but not past the throat, at dosing volumes of approximately 50 microliters (µL) adjusted for the mass of individual animals. Gentle, continuous downward pressure was applied to the dewlap of each lizard and the dose administered through the gape. Quail and mice were given oral doses via gavage. Mice used in the 40-day exposure regime were exposed to A-DNT through tainted feed rather than gavage because of the stress observed in the animals from repeated oral dosing during the subacute study. Since salamanders are sensitive to dermal exposure and experience complications from oral dosing, all exposures for salamanders were through tainted soils. An initial with a 10-d range finding study was followed by a 28-d subchronic exposure regime. Briefly, sandy loam soils (of the Sassafras variety) were passed through a coarse 5-millimeter (mm) sieve to remove debris and then air-dried on a stainless steel tray in a hood for 5 days with periodic mixing to ensure thorough drying. The soil was then sieved through a 2 mm screen and mixed to ensure uniformity and stored in a covered container. The soil was characterized as A-horizon forest soil with a loam texture comprised of 45.6 percent sand, 43.6 percent silt, 10.8 percent clay, 22.4 percent organic matter, and water holding capacity (WHC) of 23 percent. The cation exchange capacity was 7.3 centimoles per kilogram (cmol kg^{-1}), with soil pH 5.9. Compound was then dissolved in high purity acetone, and dispersed into deionized water. The solution was added to the soil, with control being mixed with only acetone. Each treatment was dried overnight in a darkened fume-hood before being mixed individually in a three-dimensional mixer for 18 hours. To prepare the individual exposures, 80 grams (g) of soil were added to 120 x 20 mm glass petri dishes, hydrated to 100 percent water holding capacity (WHC; 23 percent of soil by weight) and allowed to sit in a dark room at room temperature for 15 days prior to the start of the study. The dishes were individually weighed after 7 days and rehydrated to maintain their initial mass as necessary. Following a 15-day aging period, soil samples (about 1.5 g) were collected on days 0 (initial), 14 (midpoint), and 28 (final) from each container and pooled according to treatment. Samples were immediately frozen at -35 °C until analyzed. (Fisherbrand® is a registered trademark of Fisher Scientific Company L.L.C, Pittsburgh, Pennsylvania; Finnpipette® is a registered trademark of Labsystem On, Helsinki, Finland.)

(4) Acute Toxicity Studies. Acute oral toxicity of explosives oral DNT exposure was assessed using a stage-wise probit design (reference 2). This method integrates

three progressive dosing stages and flexibly adjusts doses and numbers of animals according to the results of the preceeding test stage. The first stage consisted of four animals of each sex each receiving a different dose. The next stage consisted of 10 animals of each sex, with test concentrations chosen at intervals where partial lethal response occurs based on the results from the first stage. The third stage used another 10 animals of each sex, focused on refining the median-lethal dose estimate and accompanying confidence intervals. In all stages, the animals were dosed once and were observed for 14 days. A lethal dose expected to result in 50 percent lethality to a population of test animals (LD_{50}), slope, and associated 95 percent confidence intervals (CIs) were determined for each sex when tested. Acute studies were integrated as part of the subacute regime for the salamander studies.

(5) Subacute Toxicity Studies. The 14-day subacute range-finding study was conducted to explore sublethal targets of toxicity and to refine exposure estimates for the subchronic study. Either 42 male lizards were randomly sorted into 7 treatment groups, or 18 quail of both sexes were sorted into 7 treatment groups, or 32 female rabbits were sorted into 8 treatment groups with the mean body weight between dose groups tested and found not to be statistically different from each other (p >0.05). Male and female mice were randomly sorted into seven groups of six each (N=42). All animals (with the exception of salamanders) were typically weighed on days -3, -1, 0, 3, 7, and 14; salamanders were weighed weekly. Single salamanders were exposed to between one and 10 concentrations of compound up to 5000 milligrams per kilograms (mg/kg) and observed daily. They were fed between 10–20 flightless fruit flies *(Drosophila melanogaster*) and qualitatively observed for evidence of feeding. Daily observations of toxicity, mortality, and behavior were recorded. Blood samples were obtained using heparinized capillary tubes following euthanasia. All animals (excepting salamanders) were then necropsied for gross pathology, and organ masses were obtained for suspected targets (e.g. brain, spleen, liver, kidney, gonads, gut, and heart).

(6) Subchronic Toxicity Studies. Sixty male lizards were randomly sorted into 6 dose groups; 120 quail consisting of 12 per sex were sorted into 1 of 5 treatments. There were 120 mice sorted to 10/treatment/sex to 1 of 6 treatments. Salamanders (20/treatment) were sorted by weight into one of five treatments (N=100). Each group in the subacute rabbit study consisted of four animals each (N=36). The mean weight between dose groups was not different for any of the groups or studies. For gavage studies, doses were administered to each animal every day for 60 consecutive days, with the exception of mice and salamanders. All animals were weighed on days -3, -1, 0, 3, 7, and weekly thereafter. Mice were exposed via contaminated feed for 40 days; salamanders were exposed for 28 days through contaminated soil. Salamanders were given flies as food, as described previously, and observed daily. Salamanders were also provided 1.5 g sphagnum moss as refugia and for environmental enrichment. Blood samples for hematological and clinical chemistry analyses were obtained as

previously mentioned, as were tissues suspected as relevant as targets (e.g., brain, liver, heart, kidneys, spleen, and testes). Tissues were removed, weighed, and fixed in a 10 percent formalin solution for histological evaluations.

(7) Gross Observations. Prominent behavioral responses, changes in feces type or occurrence, or any other unique responses were recorded and evaluated. Animals perceived as moribund and in profound stress were humanely euthanized.

(8) Hematology and Clinical Chemistries. Hematological and clinical chemistry analyses were done on blood samples from animals in the subacute and subchronic studies. A HemoCue® hemoglobin photometer was used to analyze Hgb in whole blood (output measured in g Hgb/dL). A hemocytometer was used to determine red blood cell (RBC) counts, and percent hematocrit (percentage HCT) was measured using the standard capillary centrifugation method. [and total solids (TS)]. Whole blood samples were spun using a microcentrifuge to separate cells from plasma. Plasma was used to evaluate the following clinical chemistries: albumin (ALB), alkaline phosphatase (ALP), aspartate aminotransferase (AST), calcium (Ca), cholesterol (CHOL), phosphorus, total protein (TP), triglycerides (TRIG), uric acid (URIC), globulin (GLOB), and Ca:phosphorus ratios. Plasma/serum chemistries were analyzed using the VetTest®. Additionally, if extra plasma remained, it was pooled within treatment and analyzed for testosterone concentrations using the TOSOH Bioscience AIA-360. For salamanders, only RBC, white blood cell (WBC), and Hgb were evaluated from blood samples. (HemoCue® is a registered trademark of Hemocue AB Corporation, Angelholm, Sweden; VetTest® is a registered trademark of IDEXX Laboratories, Inc., Westbrook, Maine.)

(9) Histopathology. A complete gross necropsy was performed in both the subacute and subchronic studies, including obtaining whole body and organ weights (i.e., brain, heart, liver, kidney, spleen, gonads). In the subchronic studies, organ samples were examined for histopathology after preservation in 10 percent neutral buffered formalin. Sections of organs were trimmed, placed in cassettes, embedded in paraffin, sectioned at 6 microns, stained with hematoxylin and eosin, and examined via routine light microscopy by a board-certified pathologist.

(10) Spermatozoan Analysis. Spermatozoa were collected from the epididymis and vas deferens in lizards and birds after euthanasia and immediately following dissection of tissues from the remaining urogenital tract. Briefly, spermatozoa were released by mincing the tissues with scissors into 200 µL buffered RPMI-1640 medium (Sigma Chemical, St. Louis, Missouri, USA). A volume of 2 µL of the suspension was loaded onto 20 micrometer (µm), 4-chamber slides (Leja Products B. V., Nieuw-Vennep, Netherlands) to determine cell concentration, percent motility, and progression with an Integrated Visual Optical System (or IVOS) Sperm Analyzer, Version 12.1

(Hamilton-Thorne Research, Beverly, Massachusetts, USA).

(11) Statistical Analyses. Statistical analysis was done using both Statistical Analysis System (SAS) version 9 (SAS Institute Cary, North Carolina, USA) and Sigma Stat (SPSS®) version 3.11. Initially, all data sets were tested for normal distribution using Kolmogrov-Smirnov test and Levene's test for homogeneity. If the data set failed normality tests, data were either log-transformed or nonparametric (Kruskal- Wallis) analysis was used. One-way analysis of variance was used for all normal or transformed data analysis, followed by pairwise comparison using the Tukey or Holm-Sidak methods. Survival analysis is a statistical approach for analyzing time-to-event data; in this case, time-to-death was analyzed using Sigma Stat. Specifically, the Kaplan-Meier Survival Analysis:Log-Rank command using indexed data and Holm-Sidak was selected for comparing mean values. This study was conducted under a GLP protocol approved by USAPHC (Prov) IACUC. (SPSS® is a registered trademark of SPSS, Inc., Chicago, Illinois.)

b. Salamander Studies.

(1) Red-backed salamanders (*Plethodon cinereus*) were field captured via State permit in the Gunpowder River watershed of north-central Maryland. Salamanders were individually housed in 120 x 20 mm Pyrex® glass petri dishes for a minimum 4-week acclimatization period prior to the start of TNT exposure. During acclimatization, each petri dish contained filter paper (Fisher Scientific) and sphagnum moss (about 0.5 g dry weight) moistened with deionized water to maintain humidity and provide environmental enrichment. Filter paper was changed on a weekly basis or as needed. Salamanders were fed approximately 10 uncontaminated adult mutant (wingless) fruit flies (*Drosophila melanogaster*) every 2 days. The *D. melanogaster* were obtained from The Drosophila Company (JR Tresser, Coral Spring, Florida, USA). Lighting was on a 12L/12D cycle, room temperature was maintained at 20 °C (range 18–22 °C), and relative humidity was maintained at about 50 percent. After the acclimatization period, 10–20 individual salamanders were exposed to a brief 10-day range-finding study to help determine soil exposures for the 28-d study. (Pyrex® is a registered trademark of Corning, Inc., Corning, New York.)

(2) For the subchronic 28-d study, 100 salamanders were randomly sorted into five treatment groups and exposed for 28 days (weight between groups $p > 0.95$). Sex could not be reliably determined at the initiation of treatment and was, thus, unknown. During exposure, each petri dish contained 80 g of soil hydrated to 100 percent WHC (23 percent by weight) and 0.5 g (dry weight) of sphagnum moss hydrated with 7.0 g of water. Petri dishes were individually weighed on a weekly basis and hydrated as necessary to maintain their initial water content. Salamanders were observed daily for signs of overt toxicity (e.g., lethargy, sensitivity to touch, and abnormal behavior) and

were weighed weekly to 0.001 g. Food consumption was qualitatively recorded by noting if flies remained in the dish at the next feeding event. Salamanders were weighed weekly throughout the exposures. On day 28, salamanders were anesthetized in a buffered MS-222 solution and euthanized via decapitation. Small amounts of blood, approximately 5 to 20 µL per animal, were collected for enumeration of erythrocytes and leukocytes and determination of Hgb following euthanasia. Blood was collected in heparinized microcapillary tubes from the decapitation site and discharged as a single drop onto a glass microscope slide. A 1.0 µL aliquot of blood was pipetted into a small polypropylene tube containing 19.0 µL of Natt-Herrick stain and gently mixed. Then 10 µL of the blood and stain solution was pipetted onto a hemocytometer for standard enumeration of erythrocytes and leukocytes on the same field. The remaining whole blood volume, if sufficient, was immediately used for Hgb analysis using a HemoCue Hemoglobin Photometer. The remaining body and head were preserved in 10 percent formalin. Cross sections of the entire body were taken from two levels of the head and nine levels of the body. Tissues were processed using standard histological techniques, embedded in paraffin, sectioned at 5 microns, stained with hematoxylin and eosin, and examined via light microscopy.

7. RESULTS.

a. Reptile 2,4-DNT Studies.

(1) Acute Study. The LD_{50} for male lizards was determined to be 380 mg DNT/kg with a 95 percent CI of 149 to 515 mg/kg and a slope of 4.60. The LD_{50} for female lizards was determined to be 577 mg/kg DNT with 95 percent CI of 406 to 785 mg/kg and a slope of 7.3.

(2) Subacute Study. Mortality occurred in both the 100- and 200-mg/kg-day treatments. All lizards dosed with 200 mg DNT/kg were either found dead or humanly euthanized within the first 4 days of the study. Only 2 of 6 lizards survived the 14-day study duration in the 100 mg/kg group. Lizards in the remaining dose groups – 0, 6.25, 12.5, 25 and 50 mg/kg – all survived the 14 days. Observations prior to death were similar to those of the acute study. However, no significant differences were detected between dose groups at doses less than 50 mg/kg-day with respect to changes in body weight, organ weight, food consumption, hematology, or clinical chemistries.

(3) 60-Day Subchronic Study.

(a) All lizards exposed to 0, 9, or 15 mg DNT/kg-day survived the 60-day study duration. However, dose-dependent mortality occurred in the top 3-dose groups (25, 42, and 70 mg/kg-day DNT). The control and the two lowest dose groups (9 and

15 mg DNT/kg) had 100 percent survival, unlike 25, 42, and 70 mg/kg-d, where the mean survival was 54.9±0.4, 51.4±0.4 and 39.6± 0.6 days, respectively. Lizards in the 0, 9, 15 or 25 mg/kg-day treatments all gained weight the first week of the study. However, lizards dosed with 42 or 70 mg/kg-day showed immediate weight reduction within the first few days (see Figure 1). Specifically, lizards exposed to 70 mg/kg-day lost more weight than controls from day 0 to day 7 ($p < 0.0002$), and lizards exposed to 42 mg/kg-day lost more weight than controls from day 7 to day 14 ($p < 0.001$). Lizards exposed to 25 mg/kg-day followed this trend, revealing significant weight loss relative to controls between day 14 to day 21 ($p < 0.01$). Finally, lizards exposed to 15 mg/kg-day also showed significant weight loss from controls during the final days of the study ($p < 0.042$). Analysis of food-consumption data also revealed differences. Lizards exposed to 15 mg/kg-day consumed fewer crickets per day than did control lizards. Brain, heart, liver, spleen, and testes weights were not statically different from controls; however, lizards exposed to 15 mg/kg-day had significantly larger kidneys than controls ($p < 0.029$).

(b) Clinical chemistry results revealed lizards exposed to15 mg/kg-day had a statistically significant increase in URIC and phosphorus when compared to control lizards ($p < 0.05$). No significant differences were detected in ALB, ALP, AST, Ca, CHOL, TP, TRIG, GLOB or Ca:phosphorus ratios between controls and 9 or 15 mg/kg-d dose groups. Also, no differences were found between control lizards and lizards receiving 9 mg/kg-day in either URIC or phosphorus parameters. Hematology analysis (RBC, Hgb, percent HCT, TS) did not reveal any treatment-related differences. Analysis of plasma testosterone concentrations suggests a dose-related trend (data not shown). Testosterone measurements were only obtained from a small number of lizards: control (n=8), 9 mg/kg-day DNT (n=5), and 15 mg/kg-day DNT (n=5). Thus, statistical power could not be achieved due to the small sample size.

(c) The data also suggest that lizards exposed to DNT had a dose-dependent decrease in testosterone at study termination. Histological changes related to DNT exposure included renal tubular degeneration and necrosis with resulting renal and visceral gout in the kidney. Incidences of cellular necrosis, lipogranulomas, and Kupffer cell engorgement were found in the liver; tubular degeneration and hypospermia occurred in a dose-dependent manner in the testes. Mild tubular degeneration was the most sensitive indicator of effects in the testes that was found in two individuals of the 15 mg/kg-day group, with incidence and severity increasing in lizards in higher exposure groups. The minimal to moderate kidney and liver lesions were seen at the lowest dose group (9 mg/kg-day).

(d) Following exposure to either vehicle or DNT, post-dose behavioral observations were documented daily (data not shown). Lizards exposed to 25, 40 and 70 mg/kg-day displayed arched posture significantly more frequently than control lizards

($p < 0.05$). Furthermore, lizards exposed to 25 and 70 mg/kg-day showed a significant increase in frequency of dark coloration and hanging behaviors when compared to controls ($p < 0.05$). No significant differences were found among lizards exposed to 0, 9 or 15 mg/kg-day DNT for any of these three behaviors. Additionally, no

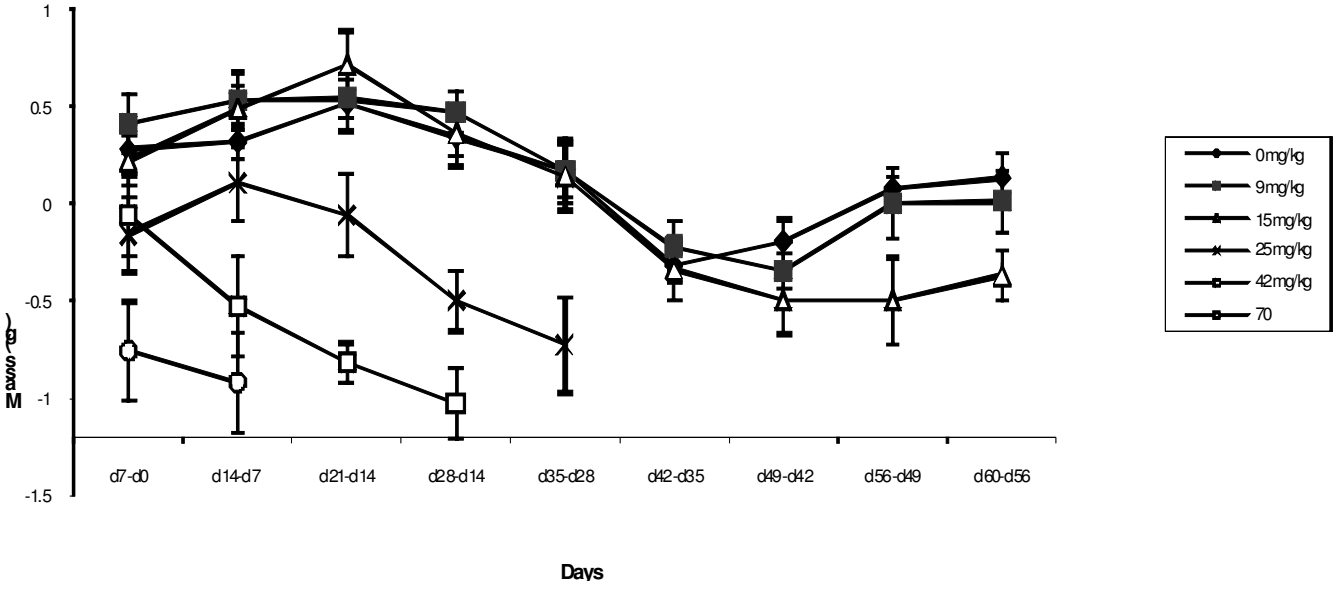

Figure 1. Mean Change in Lizard (*S. occidentalis*) Weight Following Subchronic Exposure to 2,4-DNT or Vehicle
(Bars are SEMs; Means with an asterisk are different from controls at $p < 0.05$.)

significant differences were found in hanging and dark coloration observations among lizards exposed to 40 mg/kg-day and controls.

b. Amphibian TNT Study.

(1) Since data were available from other studies in salamanders (references 3 and 4) a 10-day range finding test was not needed. TNT concentrations were short of targets in low exposures groups (Table 1). This was expected, since it is widely known that TNT is rapidly reduced in anaerobic environments at low concentrations. Trace amounts found in controls suggest misidentification of treated enclosure(s) with a control during soil sampling procedures, since it is unlikely significant cross-

contamination occurred and considering these levels are minimal. However, this could not be confirmed.

Table 1. Analytical 2,4,6-trinitrotoluene (TNT) and Primary Reduction Product Concentrations (2-amino 4,6-dinitrotoluene and 4-amino 2,6-dinitrotoluene) in Soil Based on Treatment (micrograms per gram (µg/g) dry weight).
(Means with different letters are different from each other within treatment.)

Treatment	Initial			Midpoint			Final			Mean ± SEM		
	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT
Control	0.14	0.025	0.025	0.59	0.025	0.025	1.2	0.051	0.054	0.64 ± 0.31 A	0.03 ± 0.01 A	0.03 ± 0.01 A
325 mg/kg	75	17	25	29	20	28	21	23	32	41.7 ± 16.8 A	20.0 ± 1.73 B	28.3 ± 2.03 B
750 mg/kg	450	19	23	360	30	32	310	37	39	373 ± 41.0 B	28.7 ± 5.24 B	31.3 ± 4.63 B
1500 mg/kg	1300	18	22	1200	28	30	1100	35	35	1200 ± 57.7 C	27.0 ± 4.93 B	29.0 ± 3.79 B
3000 mg/kg	2900	16	18	2600	27	28	2500	36	34	2667 ± 120 D	26.3 ± 5.78 B	26.7 ± 4.67 B

(2) Within 7 days of initiating exposure, two salamanders from the 1500 mg/kg and nine from the 3000 mg/kg died or were moribund. Two other salamanders from the 3000 mg/kg group died in the last week of exposure. Early signs of toxicity observed were lethargy and unresponsiveness to touch, though these signs were not always prevalent, and some deaths appeared sudden. Change in mean body mass relative to controls did not change until the third week of exposure (see Figure 2). By the end of exposure, salamanders in the three high exposure groups lost weight; whereas, the salamanders from the control and low concentration group did not. There was a statistically significant difference between the control and 750 and 1500 mg/kg treatments the amount of leftover (unconsumed) food/day ($p < 0.001$); however, there was no difference between control and the 3000 mg/kg group, possibly due to the smaller sample size as a result of mortalities.

(3) At necropsy, there were dose-related decreases in mean RBC concentrations and Hgb, suggesting anemia (Table 2). Highly-stained granules resembling Heinz Bodies were observed in RBCs from individuals in the two high-dose groups. Less commonly, relatively large vacuoles were also observed in RBCs from salamanders in those groups (Figure 3). No changes in mean white blood cell concentrations were found ($p > 0.18$; Table 2).

(4) Within the 1500 mg/kg and 3000 mg/kg groups, the gastrointestinal tract of all of the salamanders contained scant to a complete absence of digesta and occasionally contained a build-up of mucus. Within the 750 mg/kg group, the majority (17/20) demonstrated a similar finding. In contrast, only 3 out of 20 animals in the 325 mg/kg group were affected, and all control animals had abundant digesta. This suggests a dose-related finding likely associated with inappetence (food rejection due to poor taste). Glycogen depletion within the liver was also observed in a dose-dependent manner which is consistent with decreased feed intake. Splenic congestion was variably observed throughout the 750 mg/kg, 1500 mg/kg, and 3000 mg/kg groups. Additional histologic findings were considered to be incidental and not related to treatment.

(5) Mortality and changes in aspects of RBC structure and concentration occurred in the two high exposure groups, and changes in Hgb concentrations, body mass, inappetence, as well as splenic congestion occurred in salamanders in the 750 mg/kg (310–450 mg/kg analytical) group. No adverse effects were observed in salamanders from the 325 mg/kg group (23–75 mg/kg analytical). This suggests a conservative no-observed adverse effect level (NOAEL) and a lowest-observed adverse effect level (LOAEL) of 75 and 310 mg/kg, respectively. These data are consistent with those from earlier investigations; we recognize that differences in study design exist.

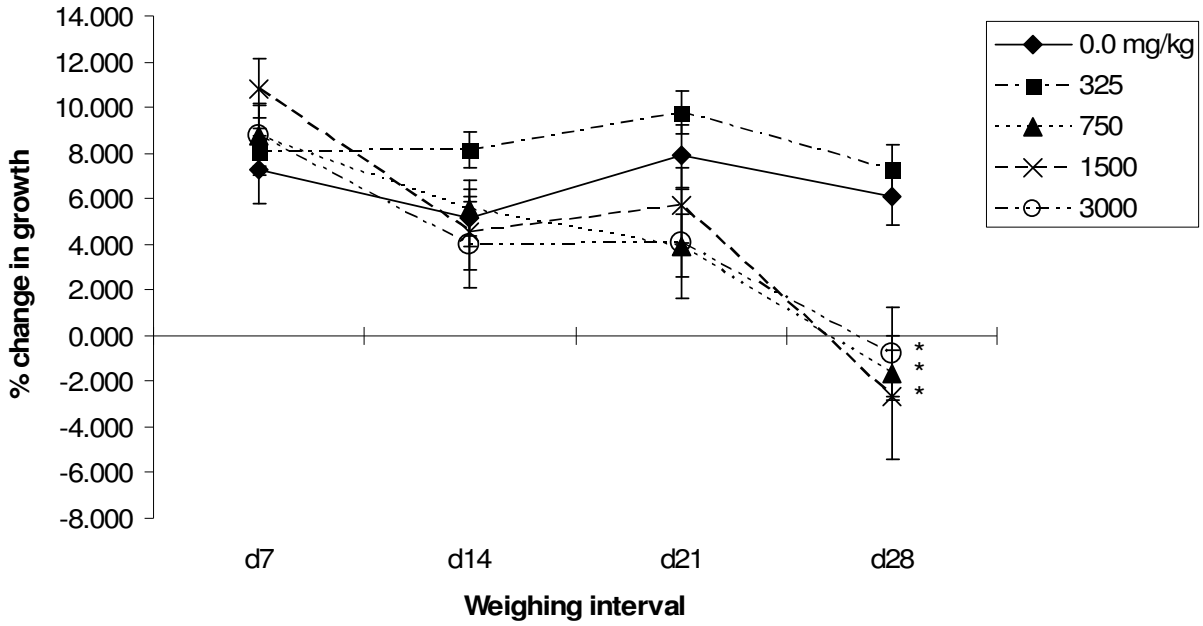

Figure 2. Mean Cumulative Change in Body Weight of *P. cinereus* Exposed to TNT in Soil Respective to Treatment

Table 2. Hematology Results from *P. cinereus* Exposed 28 Days to TNT in Soil (Means and SEM provided; means with different letters are different from each other within treatment)

Treatment	**Hemoglobin conc. (g/dL)**	***n***	**Red blood cell conc. ($x10^5$/µL)**	***n***	**White blood cell conc. ($x10^3$/µL)**	***n***
Control	6.65±0.34 A	18	1.20±0.08 A	20	4.99±0.38 A	20
325 mg/kg	6.21±0.24 AB	20	1.31±0.09 A	20	4.21±0.31 A	20
750 mg/kg	5.25±0.26 B	19	1.07±0.06 A	20	5.26±0.35 A	20
1500 mg/kg	3.09±0.38 C	18	0.68±0.07 B	18	5.59±0.50 A	18
3000 mg/kg	3.46±0.51 C	9	0.80±0.10 B	9	5.31±0.85 A	9

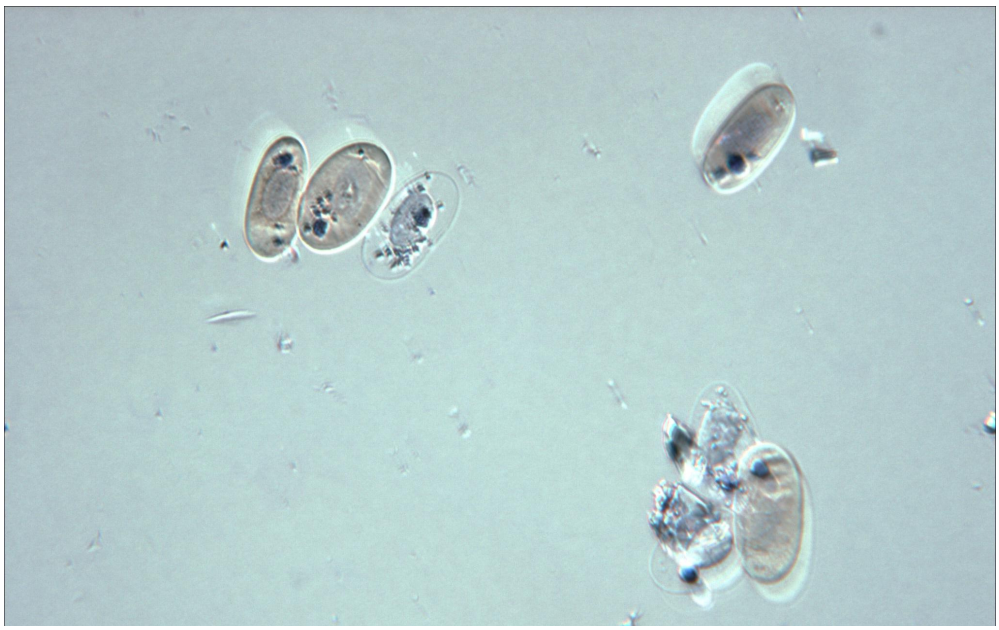

Figure 3. Red Blood Cells Showing Heinz Bodies in *P. cinereus* Exposed to TNT at 1500 mg/kg in Soil (x180)

c. Reptile TNT Study.

(1) Acute Oral Study. Overt signs of toxicity, often noted within 4 hours after dosing, included anorexia/weight loss, abdominal enlargement, lethargy/excitability/ tremor, and chromaturia with an orange-red discoloration of the area surrounding the vent. Lizards showing signs of recovery at 72 hours typically survived the 14-day observation period. All males dosed with TNT ≥ 1995 mg/kg, except one, died within 48 hours; whereas, all females dosed with ≥ 3162 mg/kg, except one, died within 48 hours. Probit analysis of the mortality data gave an estimated LD_{50} value (95 percent CI) of 1038 (332-2360 CI) and 1579 (593-3356 CI) mg/kg body weight for male and female lizards, respectively. The probit/log (dose) slope was 2.126 for females and 2.088 for males. Following dosing, females survived an average of 8.3 days, approximately 1 day more than males. There was no difference in the median lethal dose ($p > 0.05$) or survival time (Log-Rank test statistic (Z) = 0.0470; $df = 1$; $p = 0.828$) between male and female lizards.

(2) Subacute Oral Study.

(a) All individuals (N = 24) in the four highest treatment groups (132 to 1050 mg/kg-day), plus one in the 66 mg/kg-day group, died prior to the completion of the

14-day subacute study, thereby, preventing a more thorough analysis of changes in selected health endpoints. Clinical signs of toxicity (e.g., anorexia, inactivity, enlarged abdomen, tremors, and seizures) were very similar to the acute phase of the study except that severity was less intense and with a delay of onset of approximately 2 to 3 days following instigation of dosing. Lizards in the 33 mg/kg-day group were minimally affected with most of the clinical toxic responses graded at greater than or equal to 66 mg/kg-day. Mean daily cricket consumption declined notably from 33 to 66 mg/kg-day (1.71 to 0.42) and remained less than 0.63 in the higher dose groups ($F = 4.76$; $df = 5, 27$; $p < 0.005$).

(b) Chromaturia was an early consistent sign, often preceding the onset of adverse effects. Percent survival (animal days) for the control, 33, 66, 132, 263, 525, and 1050 mg/kg-day groups was 100, 100, 95, 33, 13.1, 13.1, and 7.1, respectively. Although most lizards in the higher dose groups died before the 14-day study ended, there was variation in the number of days that lizards in the different dose groups survived prior to death. The mean number of survival days for the control, 33, 66, 132, 263, 525, and 1050 mg/kg-day groups was 14, 14, 13.3, 4.7, 1.8, 1.8, and 1.0, respectively. A threshold dose occurred between 66 and 132 mg/kg-day and mean survival days decreased significantly (13.3 to 4.7; $F = 443.96$; $df = 6, 35$; $p < 0.001$) when the 66 and 132 mg/kg-day dose groups were compared. Hemoglobin ($F = 46.73$; $df = 2, 14$; $p < 0.001$) and mean cell hemoglobin concentration (MCHC) ($F = 48.16$; $df = 2, 14$; $p < 0.001$) values for the 33 and 66 mg/kg-day dose groups were decreased by approximately 22 to 61 percent and 20 to 51 percent, respectively, compared to control values. No dose-related effects were found in measures of RBC, microcytic anemia (MCV), HCT, WBC, or plasma CHOL, plasma ALB, and Ca.

(3) Subchronic Oral Study.

(a) Percent survival increased dramatically in the subchronic study when the total cumulative dose of TNT was reduced to approximately 25 percent of that of the 14-day study. Fifty-four of the sixty lizards (90 percent) dosed in the subchronic experiment survived until scheduled necropsy at the end of the study; four that died early were in the 45 mg/kg-day dose group with one each in the 35 mg/kg-day and control groups. These early deaths were preceded by a variable period of partial or complete anorexia, but generally without other signs of illness or toxicity, except in one lizard that exhibited lethargy/tremors, and in another head tilt/circling suspected to be unrelated to toxicant exposure. The lizard in the control group exhibited a sudden onset of respiratory distress that was likely the result of corn oil-toxicant aspiration.

(b) Lizards surviving to completion of the study showed dose-related effects in cricket consumption and body weight (Figure 4). Food intake was significantly reduced compared to the controls in the 35 and 45 mg/kg-day groups ($F = 10.39$; $df = 5, 54$;

$p < 0.05$), with a graded response in percent loss of body weight over the range of TNT dose, significant at 45 mg/kg-day ($F = 10.09$; $df = 5, 50$; $p < 0.001$). As with earlier TNT exposures, enlargement of the abdomen in the high-dosed groups was a frequent observation.

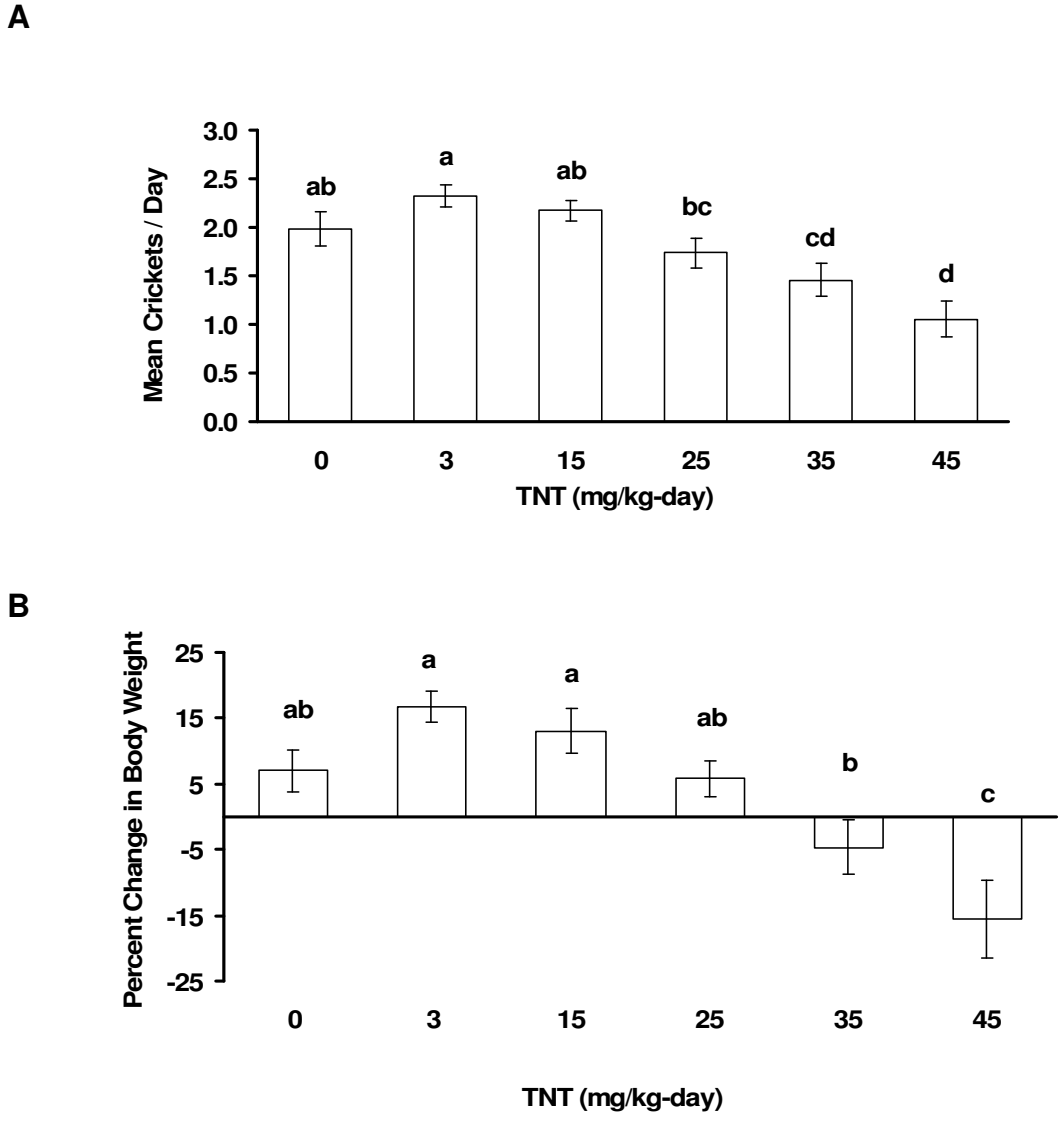

Figure 4. Mean Daily Cricket Consumption (A) and Percent Change in Body Mass; (B) in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with TNT for 60 Days (Bars are SEM; means with different superscripts are different at $p < 0.05$)

(c) A sublethal endpoint consistently observed in all dose groups and in more than 73 percent of the TNT-dosed lizards was chromaturia and an associated orange-red staining of the skin surrounding the vent (Figure 5). All lizards receiving greater than or equal to 25 mg/kg-day exhibited staining of the vent in a maximum of 7.5 days after the initiation of dosing; high-dosed animals exhibited orange staining in as few as 2 days. Chromaturia was observed in 0 percent, 31 percent, 43 percent, 38 percent, 82 percent, and 89 percent of the animals during the dosing period in the control, 3, 15, 25, 35 and 45 mg/kg-day groups, respectively. An apparent threshold was seen between the 25 and 35 mg/kg-day dose groups where the probability of observing chromaturia in treated lizards more than doubled (see Figure 6). Observing chromaturia during the 60-day exposure period was significantly greater in lizards in dose groups ≥ 35 day (F = 18.08; df = 4, 39; $p < 0.001$).

(d) The majority of lizards in the subchronic study were alert, responsive to external stimuli, and aware of their surroundings. Approximately 20 to 30 percent of animals in the 35 and 45 mg/kg-dose groups exhibited varying degrees of depression (lethargy and less responsive) and were withdrawn, particularly during the final 2 weeks of the experiment. A frequent observation in the latter stages was the ability to handle these animals with minimal resistance and/or no escape behavior. As the 60-day study progressed, the common signs of toxicity (e.g., depression, anorexia, weight loss, and chromaturia) were more frequently observed.

(e) Liver and kidney weights, measured as percent of total body weight, exhibited differences associated with TNT treatment at 45 mg/kg-day (F = 4.08; df = 5, 53; $p < 0.005$) and 35 mg/kg-day (F = 13.05; df = 5, 53; $p < 0.001$), respectively. At the highest dose level, livers were greater than 43 percent larger when compared to controls, while kidneys were greater than 170 percent larger than controls (see Figure 7). Despite the absence of a clear dose-dependent relationship, an increase in spleen/body weight percent was observed in lizards receiving 35 and 45 mg TNT/kg-day, where relative spleen weights were approximately twice that found in the control group. Brain/body weight increased progressively from 15 to 45 mg/kg-day and was different (F = 4.28; df = 5, 52; $p < 0.05$) when comparing the highest dose group to the controls (Figure 7). Organ/body weight data for the testes revealed a significant decrease in the 35 and 45 mg/kg-day groups (73 percent and 69 percent of control, respectively; F = 6.52; df = 5, 53, $p < 0.05$); testes weight was unrelated to TNT in the lower-dose range from 3 to 25 mg/kg-day (see Figure 8). When examining changes in absolute organ weights, significant differences were observed at greater than or equal to 35 mg/kg-day for the testes (decrease, F = 9.77; df = 5, 53; $p < 0.005$) and kidneys (increase, F = 7.12; df = 5, 53; $p < 0.005$) compared to the control group.

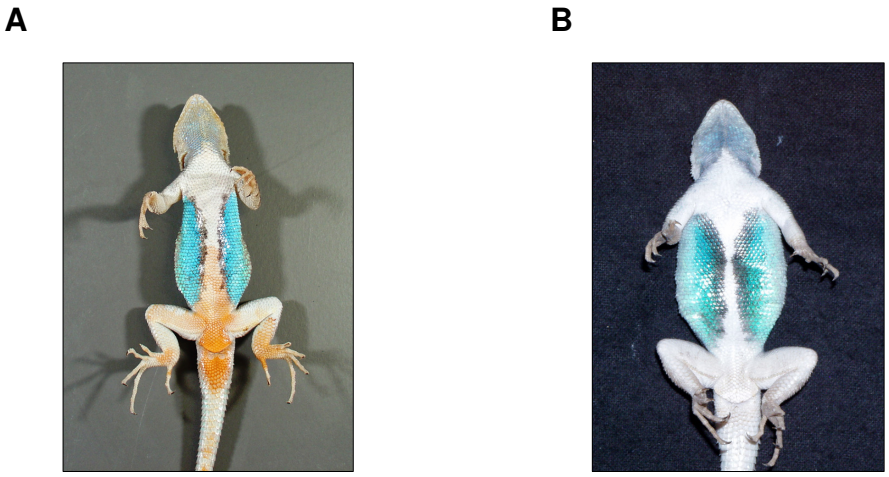

Figure 5. Observations of Chromaturia in *Sceloporus occidentalis* Exposed to TNT (left) and Corn Oil (vehicle control; right)

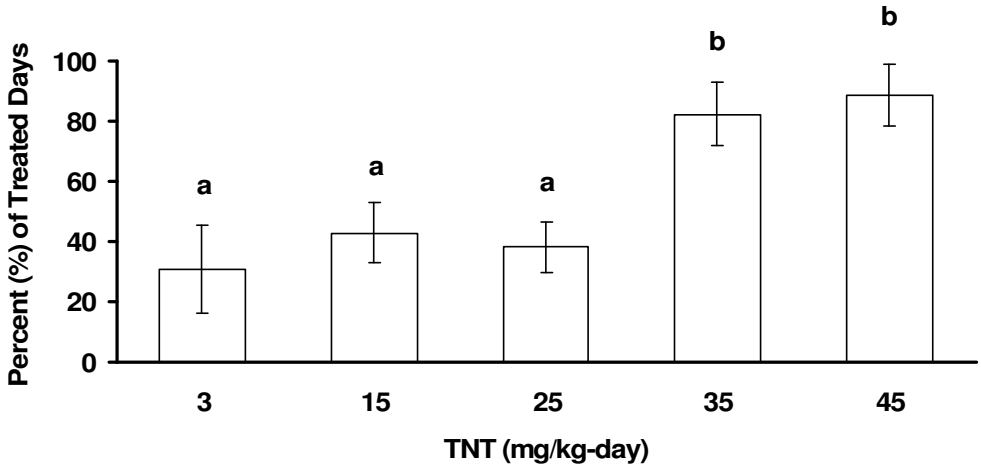

Figure 6. Percent of 60-Day Exposure Period Chromaturia was Observed Daily in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed with TNT (Bars are SEM; means with different superscripts are different at $p < 0.05$)

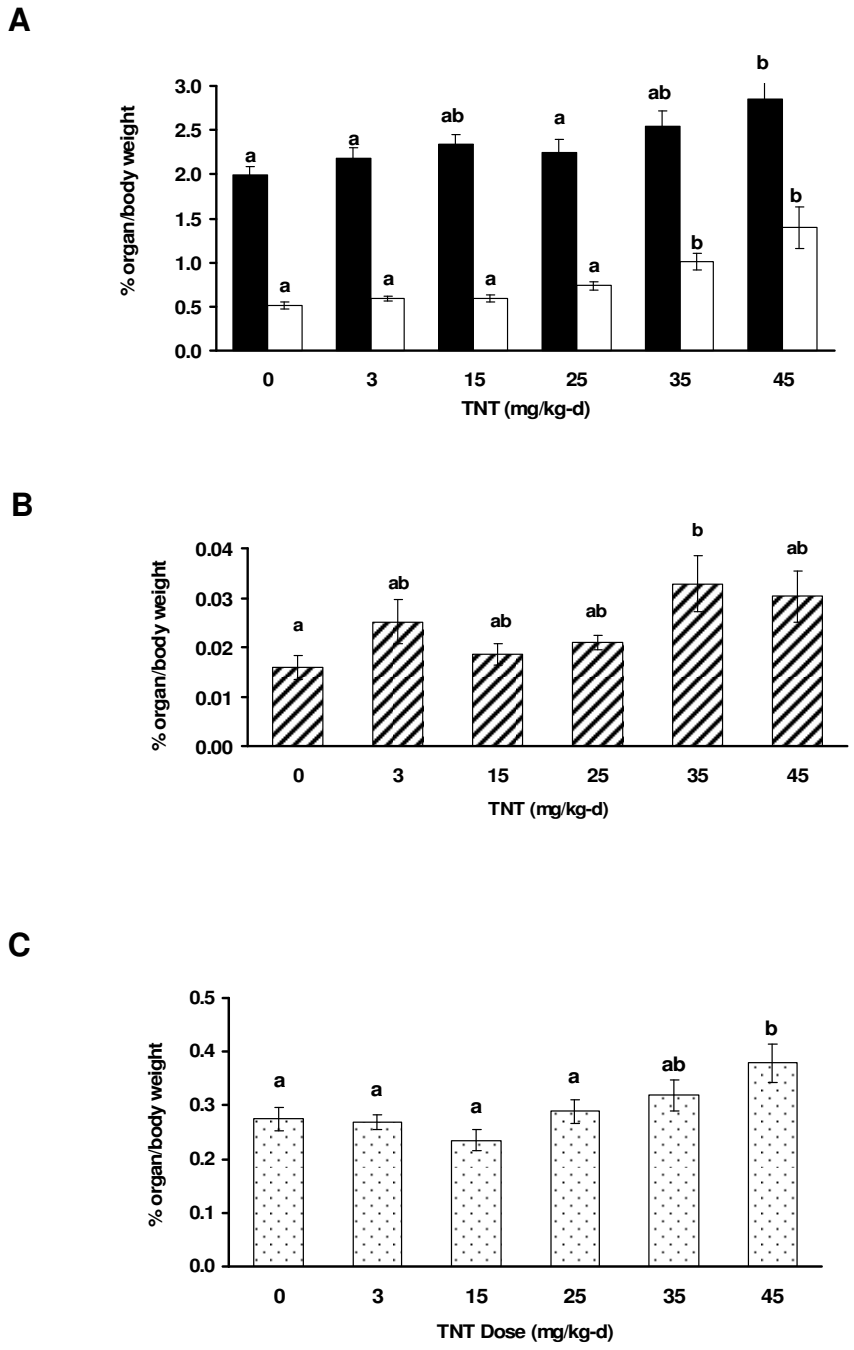

Figure 7. Changes in Relative Organ/Body Weight Percent (x100) in Liver = ■ and Kidney = □ (A), Spleen (B), and Brain (C) in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed with TNT for 60 Days
(Bars are SEM; means with different superscripts are different at $p < 0.05$)

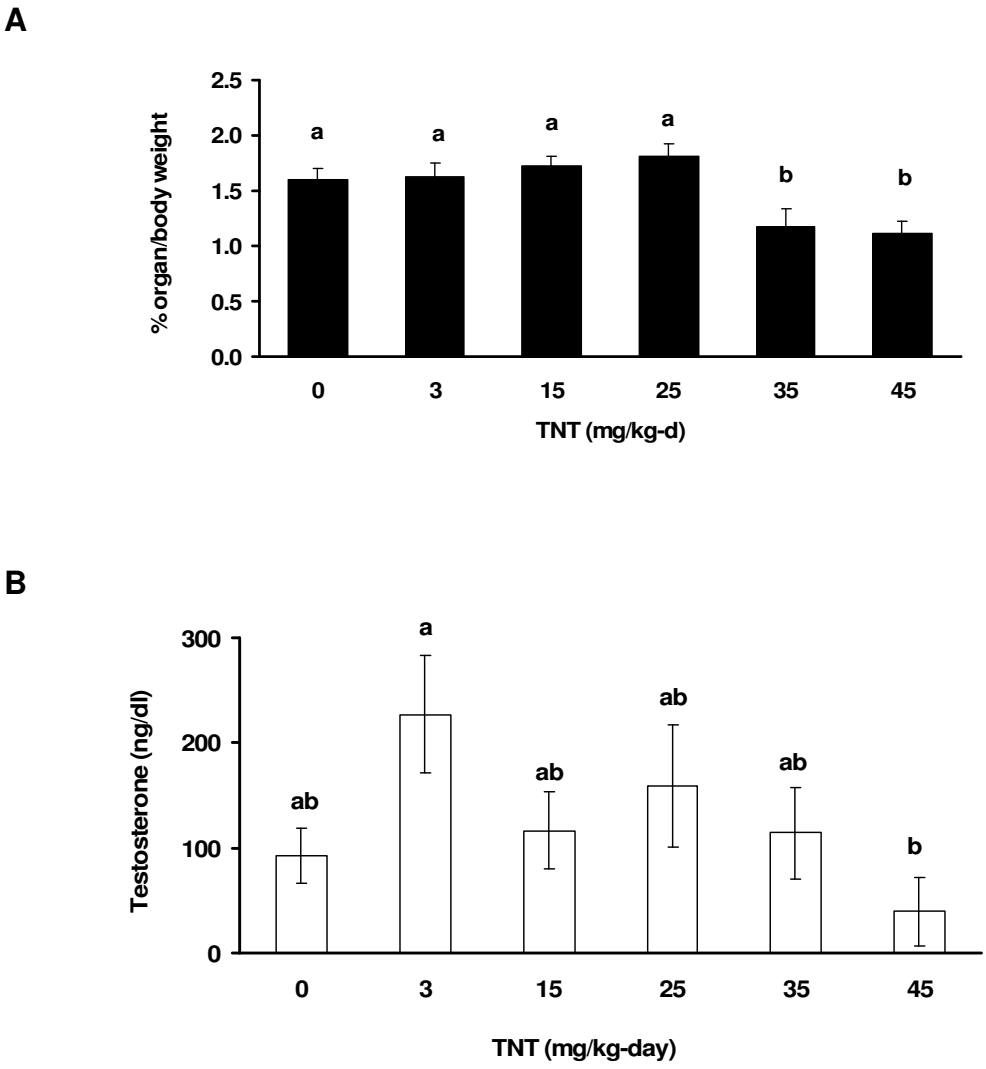

Figure 8. Changes in Relative Testes/Body Weight Mass Percent (x100; A) and Plasma Testosterone (B) as a Result of Exposure to TNT for 60 Days in the Western Fence Lizard (*Sceloporus occidentalis*)
(Bars are SEM; means with different superscripts are different at $p < 0.05$)

(f) Significant incidence of renal tubular degeneration was found in the 25 (7/10), 35 (7/10), and 45 (6/9) mg/kg-day groups. Tubular degeneration was characterized by vacuolation, cellular swelling, and increased eosinophilia. Renal

tubular necrosis in the two highest dose groups was considered a more severe manifestation of tubular degeneration with frequent accumulation of necrotic cellular debris. Renal mineralization was seen in all TNT dose groups. Hepatic Kupffer cell engorgement and multifocal lipogranulomas were identified in the greater than or equal to 15 mg/kg-day dose groups, and hepatocellular necrosis in the 25, 35, and 45 mg/kg-day groups. Kupffer cells were distended and contained variable amounts of vacuolated cytoplasm, cellular debris, and dark-brown pigment; lipogranulomas were noted to contain brown pigment as well.

(g) Changes in Kupffer cells were significantly different compared to controls in the greater than or equal to 15 mg/kg-day groups. Splenic histiocytic infiltrates were identified in the 15 mg/kg-day and higher-dose groups and presented as large, foamy, and pigment-laden macrophages. Mild to moderate testicular seminiferous tubular degeneration occurred in the 0, 3, 15, 25, 35, and 45 mg/kg-day dose groups with observations of moderate to severe hypospermia in some animals within the 35 and 45 mg/kg-day dose groups. Tubular lesions were characterized by multinucleated giant cells, disruption of orderly maturation, and/or necrotic or apoptotic cells. Testicular changes increased in severity and incidence among the TNT-treated groups compared to controls, but not significantly so ($p > 0.05$). Examination of brain and heart revealed tissues within normal limits and no TNT-related histologic changes.

(h) Hematology parameters for male western fence lizards are presented in Table 3.

(i) A treatment-related decrease was observed in Hgb, HCT, RBC, and TS concentrations; whereas, increased MCV and WBC counts were noted in lizards that received 35 and 45 mg TNT/kg-day when compared to controls. The Hgb concentration decreased 52 percent and HCT decreased 25 percent and were significantly different between controls and lizards treated at 25 mg/kg-day ($F = 15.87$; $df = 5, 48$; $p < 0.05$) and 35 mg/kg-day ($F = 4.28$; $df = 5, 48$; $p < 0.05$), respectively. The RBC counts were 23 percent lower in the 35 mg/kg-day group ($p < 0.05$) and 25 percent lower in the 45-mg/kg-day group ($p = 0.085$); fewer samples ($n = 6$) in the highest exposure group prevented statistical significance ($F = 3.77$; $df = 5, 48$).

(j) Clinical chemistry analysis of male lizards revealed that parameters examined exhibited no dose-related differences, except a decrease in total phosphorous and ALB concentrations in the 35 to 45 mg/kg-day dose groups. Most plasma chemistry results were highly variable and with minimal effects only at the higher doses (Table 4). Mean values for CHOL were lower for animals in the 3 and 35 mg/kg-day groups, though followed no-dose response pattern.

Table 3. Hematology Parameters in Male Western Fence Lizards (*Sceloporus occidentalis*) Dosed Orally with TNT for 60 Days (Data are mean ± *SEM*. Significantly different from control mean *$p < 0.05$; **$p < 0.005$)

	TNT (mg/kg-d)					
Parameters	**0 (*n* = 9)**	**3 (*n* =10)**	**15 (*n* = 10)**	**25 (*n* = 10)**	**35 (*n* = 9)**	**45 (*n* = 6)**
Hb grams per deciliter (g/dL)	9.10 ± 0.55	8.23 ± 0.29	7.67 ± 0.39	7.44 ± 0.22*	5.09 ± 0.61**	4.40 ± 0.44**
Hct (%)	33.56 ± 2.33	31.20 ± 1.40	33.20 ± 1.56	33.70 ± 1.20	26.22 ± 2.22*	25.17 ± 1.38*
RBC (10^6/µL)	1.36 ± 0.09	1.47 ± 0.08	1.42 ± 0.13	1.44 ± 0.09	1.05 ± 0.09*	1.02 ± 0.13
TS (g/dL)	6.18 ± 0.46	5.55 ± 0.26	5.88 ± 0.37	5.87 ± 0.17	4.86 ± 0.18*	4.72 ± 0.29*
WBC (10^3/µL)	22.7 ± 2.55	29 ± 3.28	22.8 ± 1.50	22.9 ± 1.27	25.4 ± 2.53	34.1 ± 6.87
MCV ((fl)	249.2 ± 15.43	216.3 ± 12.50	244.0 ± 14.60	240.6 ± 14.69	252.1 ± 12.64	263.0 ± 30.32
MCHC (g/dL)	27.31 ± 0.55	26.49 ± 0.34	23.08 ± 0.40	22.13 ± 0.28*	18.99 ± 0.85*	17.42 ± 1.33*

Table 4. Blood Chemistry in Male Western Fence Lizards (*Sceloporus occidentalis*) Dosed Orally with TNT for 60 Days (Data are mean ± *SEM;* significantly different from control mean; *$p < 0.05$; **$p < 0.005$. Globulin (GLOB) was calculated).

	TNT (mg/kg-d)					
Parameters	**0 (*n*)**	**3 (*n*)**	**15 (*n*)**	**25 (*n*)**	**35 (*n*)**	**45 (*n*)**
Total Protein (TP) g/dL	5.23 ± 0.40 (8)	4.72 ± 0.19 (9)	5.00 ± 0.29 (5)	5.30 ± 0.15 (7)	4.59 ± 0.14 (7)	3.83 ± 0.41* (3)
ALB (g/dL)	2.80 ± 0.27 (7)	2.30 ± 0.19 (8)	2.50 ± 0.27 (5)	2.58 ± 0.15 (6)	2.19 ± 0.13 (7)	1.60 ± 0.00 (1)
GLOB (g/dL)	2.70 ± 0.13 (7)	2.44 ± 0.06 (8)	2.48 ± 0.05 (5)	2.63 ± 0.06 (6)	2.41 ± 0.09 (7)	2.10 ± 0.00 (1)
Alkaline Phosphatase (ALKP) units per liter (U/L)	87.8 ± 21.31 (5)	79.14 ± 26.14 (7)	69.75 ± 9.99 (4)	59.83 ± 8.12 (6)	42.6 ± 4.75 (5)	53.00 ± 0.00 (1)
CHOL milligrams per deciliter (mg/dL)	472.33 ± 53.74 (6)	281.57 ± 47.21* (7)	391.8 ± 29.55 (5)	383.5 ± 29.49 (6)	262.00 ± 32.75* (7)	386.00 ± 0.00 (1)
Calcium (Ca) (mg/dL)	12.26 ± 0.54 (9)	12.34 ± 0.24 (10)	12.35 ± 0.17 (10)	11.67 ± 0.28 (9)	10.55 ± 0.32* (8)	11.38 ± 0.75 (6)
Phosphorus (mg/dL)	13.35 ± 0.83 (9)	13.32 0.64 (10)	12.71 ± 0.57 (10)	13.04 ± 0.76 (9)	12.81 ± 0.84 (8)	13.25± 1.47 (6)

(k) Spermatozoa counts and motility measures were variable for all treatment levels. Spermatozoa counts (expressed as number/testes mass) for the epididymis and vas deferens were not different across treatments ($F = 1.46$; $df = 5, 48$; $p = 0.221$, data not shown). Sperm counts calculated as number per vas deferens/BW ratio and number per testes/body weight ratio ranged from 55 to 66 percent of controls in the three high-dose groups, yet were not different between treatments including controls ($p = 0.218$, data not shown).

(l) The TNT exposure did not decrease plasma testosterone concentrations in a dose dependant manner (see Figure 8). High variability in testosterone, particularly in the lower doses, precluded significant differences at all treatments except for 45 mg/kg-d animals. A trend towards decreasing concentrations was observed in male lizards receiving greater than or equal to 25 mg/kg-day.

d. Mammal HMX Study.

(1) Earlier reports conducted in rodents suggested that HMX was relatively low in toxicity for those species. However, an acute study conducted in rabbits combined with information that HMX was largely not absorbed in rodents provided a possible explanation and the suggestion that mammalian species with a different gastrointestinal physiology (e.g., hind gut fermenters and ruminants) may possibly be able to better absorb HMX from oral exposures and consequently be more sensitive to HMX. Therefore, rabbits were chosen to conduct the acute and subacute studies.

(2) Rabbits orally dosed at greater than 256 mg/kg experienced clonic convulsion to include salivation roughly 21 hours post exposure and were moribund and euthanized for humane considerations. An individual orally dosed at 80 mg/kg exhibited mild convulsions, head shaking, and ataxic movements. Diarrhea was noted for 2 days, and the animal appeared to fully recover 4 days post exposure. Rabbits dosed during subsequent phases of the stagewise probit exhibited convulsions at exposures exceeding 70 mg/kg. Based on these data, an LD_{50} of 93 mg/kg (76-117 CI) was determined.

(3) During the subacute exposures, mortality or moribund sacrifice occurred in the two highest groups (4 out of 4 at 25 mg/kg and 3 out of 4 at 50 mg/kg)—the latter due largely to loss of body mass. Symptoms from exposure were largely consistent with those described from acute exposures. Seizures often began approximately 48 hours after the initial dose and were often transient. Changes in feed consumption and body mass occurred respective to treatment (see Figure 9). No changes in hematology data were attributed to treatment (see Table 5). Changes in blood urea nitrogen (BUN), creatinine (CREA), chloride, and nearly so

for cholesterol (CHOL) and aspartate aminotransferase (AST) were found predominantly associated with the two highest dose groups, though these levels were variable and at levels associated with mortality (Table 6). Incidences of seizures were intermittently observed at levels of 10 mg/kg-day and above during the study. Based on the data collected in the present study, the NOAEL and LOAEL were determined at 5 and 10 mg/kg HMX, respectively.

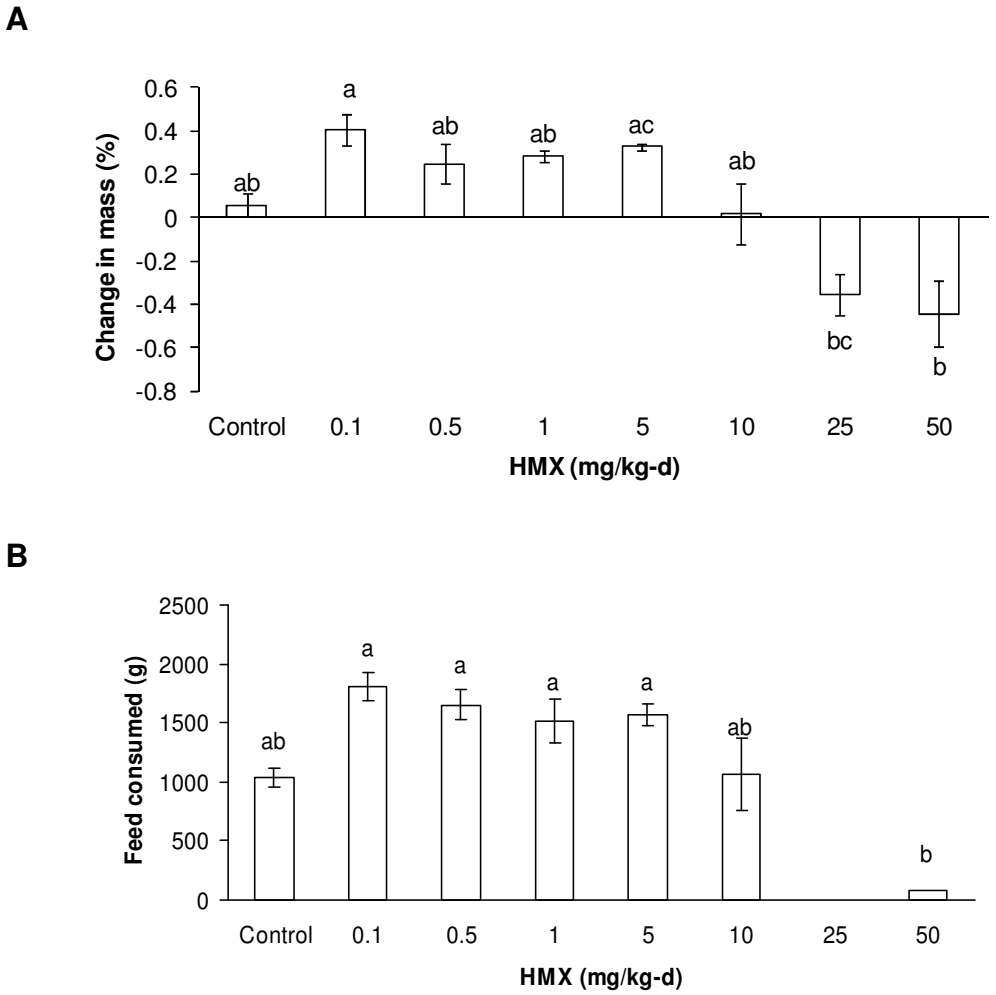

Figure 9. Mean Change in Body Mass (A) and Feed Consumption (B) Respective to Oral Dose of HMX for 14-days in the Rabbit
(Bars with different superscripts are different at $p < 0.05$)

Table 5. Hematology Results of Rabbits Exposed to Daily Oral Doses of HMX for 14 Days

Parameter	0 mg HMX/kg-d (N=4)	0.1 mg HMX/kg-d (N=4)	0.5 mg HMX/kg-d (N=4)	1 mg HMX/kg-d (N=3)	5 mg HMX/kg-d (N=4)	10 mg HMX/kg-d (N=4)	25 mg HMX/kg-d (N=1)	50 mg HMX/kg-d (N=2)	p=
MPV	4.2±0.2	4.6±0.2	4.7±0.2	4.2±0.3	4.8±0.1	4.6±0.1	5.9±0.0	4.0±0.3	0.206
HCT (%)	29.9±2.8	34.4±0.7	33.9±1.0	32.5±0.5	35.9±1.0	35.5±1.4	41.6±0.0	30.5±3.4	0.119
Hgb (g/dL)	9.9±1.1	12.0±0.1	11.5±0.3	11.0±0.1	12.3±0.4	12.2±0.4	13.9±0.0	11.0±1.2	0.130
MCV (fl)	65.8±2.7	62.5±1.4	62.3±1.1	59.9±1.1	62.0±0.6	62.7±1.1	60.0±0.0	59.6±1.5	0.218
MCH (pg)	21.7±0.6	21.9±0.7	21.0±0.4	20.3±0.3	21.2±0.3	21.6±0.7	20.1±0.0	21.5±0.5	0.451
MCHC (g/dL)	33.1±0.7	35.0±0.8	33.8±0.1	33.8±0.4	34.2±0.5	34.4±0.4	33.5±0.0	36.1±0.1	0.298
RDW	23.1±6.8	16.5±0.6	16.2±0.9	15.3±0.6	16.0±0.6	16.2±0.8	15.7±0.0	15.1±0.1	0.766
Platelet (PLT)	414.5±129.8	283.8±11.1	263.8±37.7	431.3±95.6	247.8±81.7	326.0±21.6	272.0±0.0	477.5±9.5	0.248
RBC (10^6/µl)	4.6±0.0.6	5.5±0.2	5.5±0.2	5.4±0.1	5.8±0.2	5.7±0.3	6.9±0.0	5.1±0.4	0.240
WBC (10^3/µl)	5.4 ±1.6	7.4±1.2	7.3±0.7	8.0±1.4	5.2±1.7	7.5±0.4	4.5±0	5.8±1.8	0.707
Neutrophils	35.4±11.7	13.8±1.3	14.9±2.1	19.5±6.8	18.9±9.9	15.1±2.4	91.8±0.0	86.1±1.9	0.159
Lymphocytes	52.3±12.7	82.4±3.0	78.8±3.8	75.8±5.7	74.6±14.2	79.8±3.5	2.7±0.0	6.5±1.2	0.076
Monocytes	6.9±2.1	2.9±1.4	4.3±1.2	3.2±0.7	4.1±2.7	3.2±0.8	3.6±0.0	4.6±1.7	0.695

Table 5. Hematology Results of Rabbits Exposed to Daily Oral Doses of HMX for 14 Days (continued)

Parameter	0 mg HMX/kg-d (N=4)	0.1 mg HMX/kg-d (N=4)	0.5 mg HMX/kg-d (N=4)	1 mg HMX/kg-d (N=3)	5 mg HMX/kg-d (N=4)	10 mg HMX/kg-d (N=4)	25 mg HMX/kg-d (N=1)	50 mg HMX/kg-d (N=2)	p=
Eosinophils	0.4±0.3	0.01±0.003	0.003±0.003	0.05±0.02	0.04±0.01	0.01±0.003	0.0±0.0	0.05±0.03	0.064
Basophils	5.0±2.3	0.9±0.5	2.0±0.6	1.5±0.7	2.4±1.8	1.9±0.4	1.9±0.0	2.8±1.4	0.760

Table 6. Plasma Chemistry Data of Rabbits Exposed to Daily Oral Doses of HMX for 14 Days

Parameter	0 mg HMK/kg-d (N=4)	0.1 mg HMX/kg-d (N=4)	0.5 mg HMX/kg-d (N=4)	1 mg HMX/kg-d (N=3)	5 mg HMX/kg-d (N=4)	10 mg HMX/kg-d (N=4)	25 mg HMX/kg-d (N=1)	50 mg HMX/kg-d (N=2)	p =
Sodium (Na) (mmol/L)	145.0±2.3	145.8±0.6	144.5±1.3	144.7±0.3	145.0±0.4	145.0±0.7	170.0±0.0	149.0±2.0	0.236
Potassium (K) (mmol/L)	4.4±0.2	4.3±0.1	5.8±1.3	3.8±0.7	4.5±0.4	4.3±0.2	5.2±0.0	2.9±0.1	0.271
Chloride (Cl) (mmol/L)	105.5±1.0[a]	104.0±1.0[a]	103.3±1.5[a]	105.3±0.9[a]	104.3±0.8[a]	103.8±0.9[a]	131.0±0.0[b]	115.0±2.0[c]	<0.001
ALB (g/dL)	2.9±0.1	3.4±0.1	3.2±0.2	2.9±0.1	3.2±0.05	3.2±0.1	3.6±0.0	3.0±0.2	0.099
Alanine Aminotransferase (ALT)	40.0±5.0	42.0±0.0	817.0±0.0	41.0±0.0	71.5±19.5	52.0±0.0	287.0±0.0	123.0±0.0	0.300
AST (U/L)	12.3±5.1	18.3±3.9	545.8±511.1	8.7±6.2	66.5±48.8	29.3±15.2	960.0±0.0	41.5±4.5	0.059
GLOB (g/dL)	2.4±0.1	2.0±0.1	2.1±0.05	2.1±0.1	2.0±0.03	2.0±0.05	2.3±0.0	2.1±0.1	0.146
TP (g/dL)	5.3±0.1	5.3±0.1	5.4±0.2	5.1±0.2	5.3±0.1	5.3±0.2	5.9±0.0	5.1±0.2	0.692
Glucose (GLU) (mg/dL)	146.8±3.6	157.0±5.8	150.3±7.9	161.0±12.9	150.5±8.0	144.8±5.2	397.0±0.0	164.0±4.0	0.546

Table 6. Plasma Chemistry Data of Rabbits Exposed to Daily Oral Doses of HMX for 14 Days (continued)

Parameter	0 mg HMK/kg-d (N=4)	0.1 mg HMX/kg-d (N=4)	0.5 mg HMX/kg-d (N=4)	1 mg HMX/kg-d (N=3)	5 mg HMX/kg-d (N=4)	10 mg HMX/kg-d (N=4)	25 mg HMX/kg-d (N=1)	50 mg HMX/kg-d (N=2)	p =
CHOL	97.8±23.2	65.5±12.6	73.3±7.8	177.0±50.9	63.5±6.1	67.3±8.5	279.0±0.0	278.5±42.5	0.054
Lactate dehydrogenase (LDH) (U/L)	217.3±24.1	194.5±23.1	4369.8±4100.1	152.0±12.2	425.8±246.1	317.3±110.2	3528.0±0.0	395.5±193.5	0.137
PHOS (mg/dL)	6.2±0.3	6.0±0.5	6.4±0.4	5.2±0.2	6.1±0.4	5.5±0.3	12.8±0.0	5.8±1.0	0.456
Ca (mg/dL)	11.8±0.1	11.9±0.1	11.7±0.3	10.5±1.1	11.9±0.1	12.0±0.1	10.0±0.0	10.0±1.0	0.160
BUN	11.0±0.8[a]	13.5±1.4[a]	15.3±1.6[ab]	22.0±4.0[b]	13.3±0.9[c]	12.3±1.1[ac]	106.0±0.0[d]	35.5±4.5[e]	<0.001
Creatinine (CREA)	0.7±0.06	0.7±0.0	0.7±0.05	0.8±0.0	0.7±0.0	0.7±0.03	1.5±0.0	1.0±0.2	0.021

Note: Mean values with different superscripts are different at < 0.05.

e. Amphibian HMX Study. The amphibian test began with a 10-day range-finding study. Given the lack of toxicity experienced in previous bioassays, the 10-day range-finding study consisted of three animals for each of eight treatments. Analytical HMX soil concentrations at the end of the exposure period were considerably less than target concentrations (Control = <10 mg/kg, 25 mg/kg = 14.8 mg/kg, 100 mg/kg = 94.3 mg/kg, 400 mg/kg = 314.74 mg/kg, 750 mg/kg = 638.73 mg/kg, 1200 mg/kg = 920.53 mg/kg, 2500 mg/kg = 1850.51 mg/kg, and 5000 mg/kg = 1969.01 mg/kg). The differences between target and analytically determined concentrations were attributed to the heterogeneity of the HMX amended soil and the grab sampling technique utilized. All of the salamanders survived the exposure period without any apparent effects. No change in body mass was attributable to treatment (d0-d5, p >0.34; d5-d10, p > 0.88; Figure 10). In like manner, no changes or trends were apparent in behavior, red or white blood cell concentrations, or mean hemoglobin levels (see Table 7), and no adverse histology was observed.

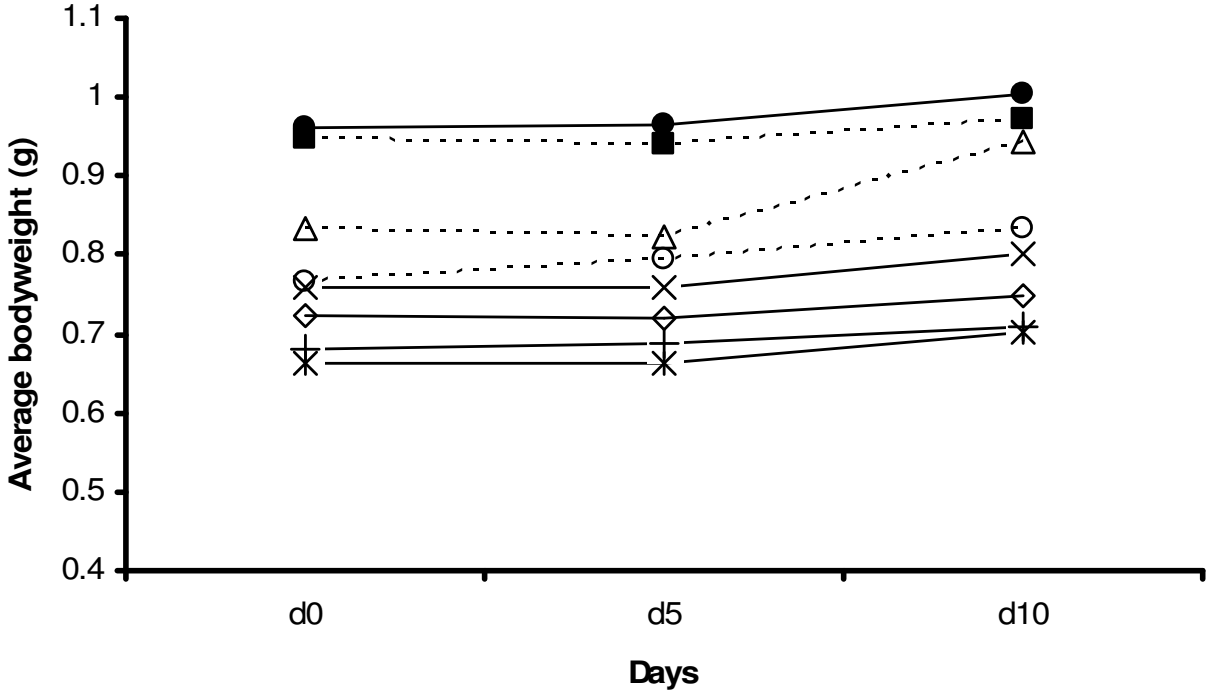

Figure 10. Mean Change in Body Mass in Red-Backed Salamanders from Exposure to HMX in Soil (Bars represent SEM; —●— Control; —×— 25 mg/kg; —+— 100 mg/kg; ---○--- 400 mg/kg; ---△--- 750 mg/kg; ---■--- 1200 mg/kg; ---∗--- 2500 mg/kg; —◇— 5000 mg/kg)

Table 7. Mean RBC, WBC, and HGB in the Red-Backed Salamander as a Result of Exposure to HMX in Soil for 10 Days

	Treatment								
Parameter	**0 mg/kg (N=3)**	**25 mg/kg (N=3)**	**100 mg/kg (N=3)**	**400 mg/kg (N=3)**	**750 mg/kg (N=3)**	**1200 mg/kg (N=3)**	**2500 mg/kg (N=3)**	**5000 mg/kg (N=3)**	**p=**
Hgb (g/dL)	7.2±0.9	7.10±0.2	8.8±0.0	6.9±0.0	5.9±0.6	7.3±1.1	6.1±3.2	7.3±1.2	0.945
RBC (10^5/µL)	1.3±0.1	1.6±0.4	1.6±0.3	1.1±0.2	1.2±0.1	1.5±0.4	9.1±0.3	1.2±0.2	0.637
WBC (10^3/µL)	4.3 ±0.1	7.5±2.6	3.5±2.1	4.5±1.5	5.9±1.1	3.9±0.1	4.3±1.0	5.5±0.3	0.791

f. Avian A-DNT Study.

(1) Acute Study. Birds dosed with 2A-DNT at less than 1190 mg/kg survived. Mixed responses occurred at levels between 1190 and 1585 mg/kg, and mortality/morbidity was consistent at doses greater than 1585 mg/kg. Signs consistent with exposure included scant white feces (urates only), lethargy, and ataxia. These responses resulted in an estimated LD_{50} of 1167 mg/kg, with a 95 percent CI of 942 to 1445 mg/kg.

(2) Subacute Toxicity. Mortality was observed in all treatment levels except for controls. All birds in the 125 mg/kg-day and above treatment groups died or were moribund within 14 days of exposure. Only one male from the 50-mg/kg-day dose group died within 14 days. Time to death was relative to dose group. Weekly body weights did not differ significantly. Since only birds from the control and 50-mg/kg-day treatment groups survived until day 14, statistics for organ:body weight ratios could only be analyzed from these two groups; no significant differences were observed for any organ:body weight ratios at day 14. Consistent signs observed in 2A-DNT treated birds at all exposure levels included enlarged gallbladders, green (bile most likely) food contents in gizzard, no food in lower gastrointestinal tract although the crop was full, and scant white feces.

(3) Subchronic Toxicity.

(a) Mortality. Only four females did not survive throughout the 60 days of exposure. One female from the 14 mg/kg-day dose group was found dead at day 49 of exposure. Upon necropsy, no gross lesions were observed; the kidneys were pale, and the gallbladder was enlarged. Two females from the 14-mg/kg-day treatment level were euthanized at days 35 and 48 of exposure to 2A-DNT due to an inability to stand. The female that was euthanized at day 35 had an edematous gastrointestinal tract with milky, green food contents in the gizzard and pale kidneys. The female that was euthanized at day 48 of exposure exhibited slight tremors when handled and was observed to have opaque pericardial effusion (~0.9 mL) opaque, very liquid exudate), green food contents in the gizzard, and ovaries that appeared to be regressing. The one female from the 30 mg/kg-day treatment group that was found dead at 48 days of exposure had an edematous gastrointestinal tract and a small, white patch on one lobe of the liver; histological findings observed in this bird were limited by moderate tissue autolysis. All males survived throughout the 60 days.

(b) Feed Consumption and Body Weights. Males in the control group consumed significantly less feed than the individuals from the 2A-DNT treated groups at day 4 (Table 8), and males from the control and 30 mg/kg-day treatment groups consumed less feed than members of the 0.5, 3, and 14 mg/kg-day groups at days 39 through 60. Females exhibited similar trends of decreased feed consumption in the 0 and 30 mg/kg-day treatment levels compared to the others, but this was only observed for days 4 through 25. Changes in body weight did not correspond to significant alterations in feed consumption (Table 9); body weight remained similar among the treatment levels throughout the entire study in both genders.

i. No significant differences in organ:brain weight ratios were observed for heart, kidney, spleen, or gonads for either gender; however. liver:brain weight ratios were affected by 2A-DNT in both sexes (Table 10). Males from the 30-mg/kg-day treatment group had the largest mean liver:brain weight ratio, but it was only significantly different than that of the 0.5-mg/kg-day dose group.

ii. Similarly, the average liver:brain weight ratio in females was largest in the 30 mg/kg-day treatment group; however, it was only significantly larger than those of the 0.5, 3, and 14 mg/kg-day groups. Brain:body weight ratios did not differ among treatments.

(c) Cellularity and Plasma Chemistries. Total WBC counts decreased in a dose-dependent manner in females only (Table 11). Female total leukocyte counts from birds exposed to 30 mg 2A-DNT /kg-day were significantly different than controls. No other measures of cellularity were significantly altered in either gender.

i. In male bobwhite, only levels of triglycerides were affected by 2A-DNT exposure (Table 12). Plasma triglyceride levels from birds exposed to 0.5, 3, and 14 mg 2A-DNT/kg-day were significantly higher than birds from the control and 30 mg/kg-day dose groups.

ii. Specifically, levels of plasma triglycerides from birds receiving 0.5 and 3 mg 2A-DNT/kg-day were higher than controls and levels between the control and 30 mg/kg-day treatment groups did not differ. No significant differences in plasma chemistry measures were observed in female Northern bobwhite.

(d) Histopathology. Hepatocellular vacuolation was observed in control group males and females, 14 mg/kg-day group females, and 30 mg/kg-day group males and females. The overall incidence of hepatocellular vacuolation was not remarkably different between the 0-mg/kg control group males and females and the 30 mg/kg-day group males and females. The overall severity of the hepatocellular

vacuolation was slightly increased in the 30 mg/kg-day group animals compared to the control animals, although two control females had hepatocellular vacuolation present with the same severity (moderate and severe) as observed in some 30 mg/kg-day group females. Also, splenic reticular cell hyperplasia was noted in one 30-mg/kg-day group male and two 30 mg/kg-day group females. The individual spleens with reticular cell hyperplasia did not directly correlate to the highest individual spleen weights. Remaining histological changes were considered sporadic or naturally occurring background lesions in this species. None of the histological findings were considered to be attributed to exposure to 2A-DNT.

Table 8. Weekly Mean Feed Consumption with SEM for Male and Female Northern Bobwhite (*Colinus virginianus*) Exposed Daily to Oral Gavages of 2A-DNT (mg/kg-day; Significant differences are indicated by superscripts)

Male weekly feed consumption (g)

2A-DNT	day 4		day 11		day 18		day 25		day 32		day 39		day 46		day 53		day 60	
(mg/kg-d)	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
0	41.5	10.8	92.9	11.2	83.3[a]	5.1	98.1	11.1	92.8	6.5	85.8[a]	6.9	88.0[a]	4.5	83.2[a]	3.3	85.8[a]	2.9
0.5	45.9	6.8	98.0	5.6	103.4[b]	4.9	110.2	4.6	115.1	7.3	112.7[b]	6.5	106.8[b]	5.0	103.2[b,c]	2.7	108.5[b,c]	1.9
3.0	66.2	7.4	113.6	7.6	109.3[b]	4.7	126.0	12.0	110.4	7.7	113.5[b]	9.1	120.2[c]	7.6	107.7[b]	6.0	114.8[b]	8.1
14.0	42.1	5.3	102.3	8.6	109.8[b]	8.0	113.1	8.2	113.3	9.5	106.1[a,b]	13.0	101.1[a,b]	7.3	94.6[a,c]	3.8	100.1[b,c]	3.8
30.0	42.1	5.0	90.2	6.4	103.4[b]	7.2	99.8	5.2	98.6	6.0	95.3[a,b]	3.7	91.1[a,b]	3.6	88.9[a]	4.0	95.8[a,c]	5.0

Female weekly feed consumption

2A-DNT	day 4		day 11		day 18		day 25		day 32		day 39		day 46		day 53		day 60	
(mg/kg-d)	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
0	40.4[a]	4.3	90.4[a]	5.2	97.5[a]	5.4	109.3[a]	7.4	131.8	8.3	118.8	6.5	144.8	9.1	136.5	6.1	134.8	8.6
0.5	53.8[b]	4.1	105.3[a,b]	11.8	134.2[b]	4.1	147.3[b]	7.0	153.5	6.6	159.8	10.5	169.4	11.6	145.8	9.0	153.2	7.3
3.0	56.2[b]	5.7	107.2[a,b]	6.4	119.8[a,b]	9.0	129.8[a,b]	8.5	137.9	7.8	135.1	9.5	143.3	7.7	152.3	13.1	50.5	8.4
14.0	56.5[b]	3.8	125.8[b]	7.8	145.6[b,c]	11.6	156.8[b]	12.9	160.5	17.9	150.1	13.1	146.4	14.4	174.3	16.9	160.6	11.0
30.0	44.9[b]	3.2	95.9[a]	4.2	119.5[a,b]	8.2	149.2[b]	13.0	146.5	10.7	137.8	11.8	143.7	12.2	144.2	11.7	158.3	11.0

Notes: Means with different superscripts are different at $p < 0.05$.

Table 9. Weekly Mean Body Weight Measures with SEM for Male and Female Northern Bobwhite (*Colinus virginianus*) Exposed Daily to Oral Gavages of 2A-DNT (mg/kg-day)

Male weekly body weight (g)

2A-DNT	day 4		day 11		day 18		day 25		day 32		day 39		day 46		day 53		day 60	
(mg/kg-d)	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
0	202.5	3.7	203.8	4	204.8	3.5	208.8	3.6	208.4	4.1	208.8	4.4	210.8	4.1	212.2	4.4	212.8	4.6
0.5	208.2	3.3	205.7	3.2	207.8	3.7	208	3.2	208.8	3.3	208.8	3.4	209.3	3.8	209.2	3.8	209.3	4.3
3.0	208	5.4	208.3	5.5	209.3	5.4	211.4	5.7	211	5.8	210.3	6.3	211.3	6	210.7	6	210.3	5.9
14.0	204.8	6.4	206.8	6.4	210.8	6.6	211.8	6.9	214.5	6.5	214.9	6.7	216.9	6.5	216.8	6.4	217.3	6.5
30.0	203.6	3.1	204.8	3.7	208.3	4.2	210.8	4.4	213.8	4.7	212.5	4.4	214.3	4.2	215.1	4.2	213.8	4.2

Female weekly body weight (g)

2A-DNT	day 4		day 11		day 18		day 25		day 32		day 39		day 46		day 53		day 60	
(mg/kg-d)	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
0	222.8	4.1	227.7	4.3	225.3	5.0	224.2	5.3	228.0	4.8	228.3	5.4	231.3	5.9	235.3	5.5	230.1	6.0
0.5	219.8	4.7	217.6	4.9	220.2	4.5	220.8	4.8	220.8	5.3	218.8	4.6	221.7	4.7	217.1	4.8	218.3	4.5
3.0	223.5	4.8	223.3	4.9	222.0	5.0	220.2	5.3	224.3	4.7	222.9	6.7	224.1	5.6	222.2	5.7	224.2	5.5
14.0	232.0	4.9	236.5	4.7	236.6	4.7	237.0	5.4	233.6	5.8	235.0	5.1	228.8	6.8	230.4	8.0	229.3	8.1
30.0	230.8	5.3	232.5	4.7	232.9	4.6	231.8	5.3	230.7	6.3	230.5	4.7	232.4	5.6	232.0	5.0	232.4	4.5

Table 10. Organ:Body Weight Ratios with SE for Liver, Heart, Kidneys, Spleen, and Gonads for Male and Female Northern Bobwhite (*Colinus virginianus*) Exposed Daily to Oral Gavages of 2A-DNT (mg/kg-day) (Values with different superscripts are different at $p < 0.05$).

	liver		heart		kidneys		spleen		gonads	
males	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
0	2.47 [a,b]	0.16	1.05	0.03	1.04	0.05	0.05	0.00	1.40	0.12
0.5	2.46 [a]	0.20	1.05	0.05	1.00	0.04	0.05	0.00	1.46	0.10
3	2.41 [a,b]	0.07	1.00	0.03	1.00	0.03	0.05	0.01	1.46	0.08
14	2.74 [a,b]	0.14	1.09	0.04	1.00	0.03	0.05	0.01	1.56	0.11
30	2.84 [b]	0.14	1.08	0.03	1.10	0.03	0.05	0.01	1.53	0.09

	liver		heart		kidneys		spleen		gonads	
females	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
0	5.22 [a,b]	0.43	1.00	0.04	1.64	0.07	0.05	0.00	4.91	0.62
0.5	4.47 [b]	0.23	0.95	0.02	1.62	0.06	0.05	0.00	5.53	0.40
3	4.48 [b]	0.23	0.90	0.03	1.52	0.07	0.05	0.00	5.05	0.41
14	4.23 [b]	0.26	0.92	0.06	1.63	0.13	0.04	0.00	4.07	0.68
30	6.23 [a]	0.33	0.94	0.04	1.78	0.09	0.06	0.00	4.80	0.53

Notes: Means with different superscripts are different at $p < 0.05$.

Table 11. Mean Hematological Data (and SEM) for *Colinus virginianus* from Oral Exposures to 2A-DNT (Values with different superscripts are different at $p < 0.05$)

		HB (g/dL)		Hct (%)		RBC (10^6/µL)		WBC (10^3/µL)	
RDX (mg/kg-d)	**Gender**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
0	Male	15.28	0.40	44.92	0.96	3.77	0.17	14.71	1.54
	Female	11.11	0.40	33.10	0.90	3.14	0.13	21.26 [a]	2.43
0.5	Male	15.30	0.31	46.08	0.50	3.78	0.10	14.25	0.91
	Female	11.23	0.31	33.46	0.92	3.20	0.14	20.21 [a]	1.27
3	Male	15.19	0.27	44.42	0.87	3.84	0.14	14.58	1.05
	Female	11.17	0.50	33.67	1.40	2.99	0.14	15.72 [a,b]	1.79
14	Male	15.63	0.25	46.58	0.74	4.02	0.08	15.05	1.20
	Female	10.21	0.67	30.89	2.05	2.88	0.13	18.06 [a,b]	2.07
30	Male	15.25	0.33	44.27	1.10	3.91	0.12	15.61	2.20
	Female	11.10	0.34	33.18	0.95	2.96	0.14	12.52 [b]	1.70

Notes: Means with different superscripts are different at $p < 0.05$.

Table 12. Mean Plasma Chemistry Data (and SEM) for *Colinus virginianus* from Oral Exposures to 2A-DNT (Values with different superscripts are different at $p < 0.05$)

		ALB (g/dL)		ALKP (U/L)		ALT (U/L)		AST (U/L)		Ca (mg.dL)		CK (U/L)		GLU (mg/dL)		LDH (U/L)	
Treat-ment	**Sex**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**
0	M	1.15	0.08	149.40	25.68	21.30 [a,b]	6.98	452.10	51.89	10.14	0.09	2142.11	281.78	349.60	22.12	1150.89	389.32
	F	1.23	0.11	846.17	131.94	10.25	0.52	515.00	53.73	14.84	1.44	2118.91	365.97	501.83	55.05	730.00	83.46
0.5	M	1.15	0.03	126.75	12.05	22.17 [a]	2.85	562.33	43.97	10.11	0.07	3159.25	906.34	344.00	11.37	1119.75	222.18
	F	1.10	0.08	734.64	137.58	11.46	1.27	493.55	20.76	13.03	1.07	2502.09	302.59	526.36	58.31	731.64	73.26
3	M	1.28	0.09	151.58	18.61	12.33 [b]	1.55	518.00	40.66	10.30	0.10	1770.18	451.64	321.25	7.20	2068.08	1342.64
	F	1.00	0.06	906.67	117.44	11.00	0.97	543.42	45.00	13.54	1.45	3886.25	841.56	585.58	53.65	1258.83	221.85
14	M	1.26	0.08	165.08	23.26	13.00 [a,b]	1.56	469.33	17.42	10.21	0.12	1484.83	246.91	327.67	7.73	641.92	56.16
	F	0.99	0.10	1096.75	193.54	13.00	2.14	574.00	52.84	12.53	0.57	2861.00	724.16	525.00	71.86	1148.88	274.67
30	M	1.12	0.08	143.36	27.16	15.55 ~~[a,b]~~	2.22	512.91	54.16	10.12	0.12	2768.36	783.36	320.82	13.14	941.36	134.86
	F	1.11	0.06	577.23	119.51	11.55	1.06	447.46	48.36	14.09	0.94	2905.10	995.96	108.82	65.31	1275.73	276.80

Table 12. Mean Plasma Chemistry Data (and SEM) for *Colinus virginianus* from Oral Exposures to 2A-DNT (Values with different superscripts are different at $p < 0.05$ (continued)

		ALB (g/dL)		**ALKP (U/L)**		**ALT (U/L)**		**AST (U/L)**		**Ca (mg.dL)**		**CK (U/L)**		**GLU (mg/dL)**		**LDH (U/L)**	
Treat-ment	**Sex**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**	**mean**	**SE**

		PHOS (mg/dL)		TP (g/dL)		URIC (mg/dL)		GLOB (g/dL)		Na (mmol/L)		K (mmol/L)		Cl (mmol/L)		TRIG (mg/dL)	
treatment	F	mean	SE	mean	SE	mean	SE	Mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
0	M	5.24	0.37	4.24	0.12	3.24	0.31	3.11	0.06	162.44	6.82	5.22	0.77	122.44	0.67	117.40 [a,c]	8.80
	F	6.15	0.47	4.45	0.26	3.88	0.36	3.20	0.16	155.58	2.13	4.52	0.15	120.42	1.44	1047.42	175.19
0.5	M	4.95	0.36	4.04	0.07	4.63	0.40	2.92	0.05	162.67	1.73	5.18	0.55	121.92	0.45	176.08 [b]	17.10
	F	5.73	0.35	4.16	0.17	5.06	0.88	3.07	0.10	157.18	1.68	5.49	0.76	119.36	1.42	588.55	97.92
3	M	4.36	0.33	4.36	0.14	4.02	0.28	3.08	0.07	160.75	2.76	4.56	0.46	121.67	0.63	157.67 [b,c]	16.08
	F	6.83	0.79	4.06	0.08	4.94	0.65	3.05	0.05	158.33	2.12	5.74	1.00	119.75	1.30	833.83	125.40
14	M	4.23	0.20	4.34	0.14	3.93	0.33	3.10	0.07	158.58	2.78	4.23	0.24	121.42	0.69	142.75 [a,b]	15.26
	F	5.36	0.42	4.15	0.22	4.15	0.50	3.15	0.12	156.13	1.91	5.20	0.98	119.75	1.63	1004.00	225.05
30	M	4.35	0.24	4.08	0.15	3.45	0.36	2.95	0.08	158.33	2.95	4.38	0.50	122.92	0.51	102.91 [a]	22.95
	F	7.26	0.44	4.25	0.18	4.86	0.53	3.17	0.09	155.73	2.13	6.33	0.89	116.82	1.75	1264.64	219.80

Legend:
M – males
F – females
Notes: Means with different superscripts are different at $p < 0.05$.

g. Reptile HMX Study.

(1) Acute Study. Two out of five male lizards orally dosed at 5000 mg/kg died at 1 and 2 days post exposure. One of the males exhibited seizures and became very inactive at approximately 24 hours and died shortly thereafter. Although renal inflammation was observed, advanced autolysis of the tissue precluded evaluation of potential tubular insult. The cause of death could not be determined in the remaining animal.

(2) Overt Observations. One female dosed at 5000 mg/kg had episodes of paddling and hyperactivity with apparent complete recovery by day 5. Except for varying degrees of inactivity and an enlarged abdomen in a single female in the high dose group, remaining males and females survived without incident. Given these observations, it was concluded that the oral LD_{50} for HMX in lizards was greater than 5,000 mg/kg; therefore, satisfying the criteria for the limit test; further testing was not indicated.

h. Amphibian A-DNT Study.

(1) Concentrations of A-DNT in soil were generally lower than targets; measured mean concentrations were less than 0.050, 34, 173, 603, and 1533 mg for 0, 50, 250, 850, and 1800 mg 2A-DNT/kg soil dry weight, respectively (Table 13). Survival was 100 percent across all treatments with the exception of one death in the 603 mg/kg treatment on day 27. No changes in mean body weight were attributed to treatment at all periods during exposure ($p > 0.91$ for all comparisons; Table 14). Adverse effects observed in the two highest treatments were lethargy, unresponsiveness, difficulty and inability to right, inappetence, and a significantly greater tendency to avoid the soil and reside on the moss provided for environmental enrichment (Figure 11).

(2) The histological analyses revealed no test-article-related findings. No treatment related changes were found in Hgb or RBC concentration (RBC; $p > 0.10$); however, small changes in WBC concentration was increased and decreased relative to controls in various treatments; these changes were not consistent with increased A-DNT exposure, therefore, lack a dose-response relationship (Table 15). These data suggest that a 2A-DNT soil concentration of and exceeding 603 ± 102 mg/kg results in inappetence, soil avoidance, lethargy, unresponsiveness, and adversely impacts righting ability. Hematology analyses suggest that a concentration of and exceeding 1533 ± 33 mg/kg may result in reduced total WBC count. No adverse effects were observed at 173 ± 15 mg/kg.

Table 13. Analytical Results for 2A-DNT in Soil (mg/kg dry weight)

Target Concentration (mg/kg)	Initial	Midpoint	Final	Mean ± SEM
Control	<0.050	<0.050	<0.050	<0.050
50	37	32	33	34 ± 1.5
250	200	170	150	173.3 ± 14.5
850	400	700	710	603.3 ± 101.7
1800	1500	1500	1600	1533.3 ± 33.3

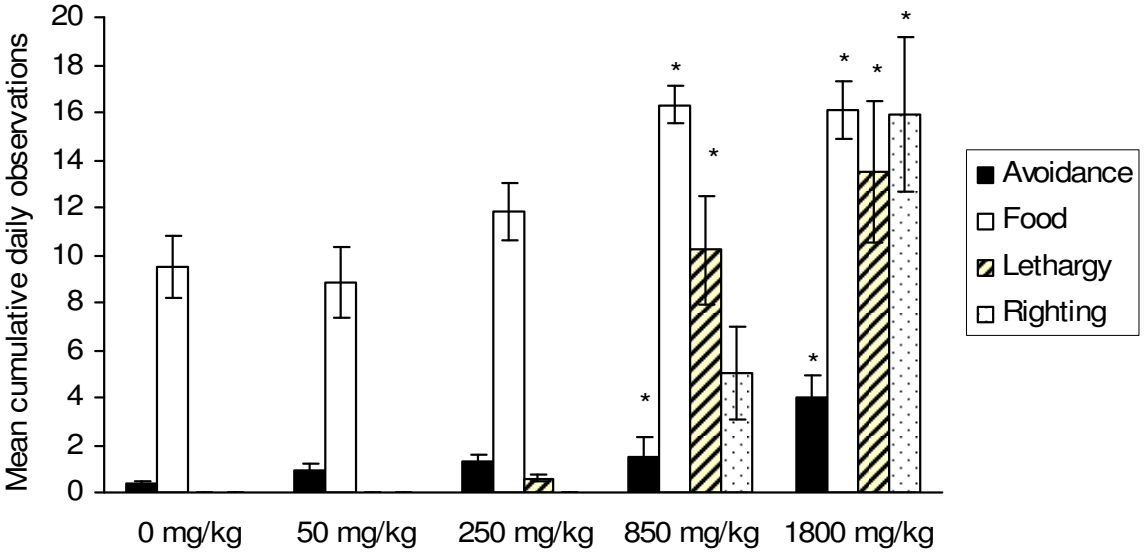

Figure 11. Mean Cumulative Observations of *P. cinereus* Residing on Moss (Avoidance), Enclosures Containing Flies (Food consumption), Lethargy, and Inability of Righting Reflex Relative to Soil Exposures of A-DNT for 28 Days (* = indicates different from control at $p < 0.05$, Kruskal-Wallis one-way Analysis of Variance (or ANOVA) on Ranks; means and SEMs provided).

Table 14. Mean Body Mass Relative to Treatment and Observation Period of *P. cinereus* from Soil Exposures of A-DNT[a]

Treatment (mg/kg)	Day 0	Day 7	Day 14	Day 21	Day 28
Control	0.78 ± 0.044	0.79 ± 0.046	0.77 ± 0.044	0.76 ± 0.043	0.77 ± 0.041
0.0	(n = 20)	(n = 20)	(n = 20)	(n = 20)	(n = 20)
50	0.79 ± 0.046	0.79 ± 0.046	0.79 ± 0.046	0.78 ± 0.044	0.79 ± 0.046
	(n = 20)	(n = 20)	(n = 20)	(n = 20)	(n = 20)
250	0.78 ± 0.045	0.78 ± 0.044	0.79 ± 0.043	0.77 ± 0.042	0.79 ± 0.044
	(n = 20)	(n = 20)	(n = 20)	(n = 20)	(n = 20)
850	0.77 ± 0.043	0.78 ± 0.046	0.78 ± 0.046	0.77 ± 0.048	0.76 ± 0.048
	(n = 20)	(n = 20)	(n = 20)	(n = 20)	(n = 20)
1800	0.80 ± 0.054	0.81 ± 0.043	0.83 ± 0.049	0.79 ± 0.045	0.76 ± 0.047
	(n = 20)	(n = 20)	(n = 20)	(n = 20)	(n = 19)

Note:
[a] Combined data for males and females

Table 15. Hemoglobin, Total RBC, and Total WBC Concentrations from *P. cinereus E*xposed 28 Days to 2A-DNT in Soil (Means and SEM provided)

Treatment (mg/kg)	Hemoglobin[a] (g/dL)	RBCs[a] ($10x^4$ cells/µL)	WBCs[a] ($10x^3$ cells/µL)
Control 0.0	8.7 ± 0.29 (n = 20)	12.82 ± 0.75 (n = 20)	4.02 ± 0.34 [b] (n = 20)
50	8.9 ± 0.30 (n = 20)	13.29 ± 0.70 (n = 20)	4.29 ± 0.55 [b] (n = 20)
250	8.6 ± 0.30 (n = 20)	12.58 ± 0.63 (n = 20)	4.35 ± 0.44 [c] (n = 20)
850	9.0 ± 0.33 (n = 19)	15.22 ± 0.76 (n = 19)	3.08 ± 0.28 [c] (n = 19)
1800	8.6 ± 0.25 (n = 19)	14.20 ± 0.97 (n = 19)	2.61 ± 0.20 (n = 18)

Notes:
[a] Combined data for males and females.
[b, c] Means with different letters are different from each other within treatment.

i. Mammalian A-DNT Study.

(1) Acute Study. Probit analysis estimated oral LD_{50} values (95 percent CI) for female white-footed mice in corn oil (gavage) as 4019 (3861-4183 CI) mg/kg for 2A-DNT and 4203 (3866-4569 CI) mg/kg for 4A-DNT; probit/log (dose) slope was 53.2 for 2A-DNT and 34.212 for 4A-DNT. At the higher doses with both compounds, and often within 4 to 6 hours, mice exhibited varying degrees of weakness, depression, ataxia, and muscle fasciculation.

(2) Subacute Study. A 14-day subacute oral gavage study followed using the slightly more toxic 2A-DNT compound in corn oil in both male and female mice at 0, 31, 49, 78, 123, 196, and 310 mg/kg-day. The dose-response relationship between food consumption and body weight changes was not statistically significant; there were no interactive effects of the two parameters sex and dose. No abnormalities were observed in the hematologic indices; plasma albumin was the only chemistry that was different and was reduced only in the 310-mg/kg-day group. Survival was approximately 98 percent with minimal clinical signs at the selected doses.

(3) Subchronic Study.

(a) Concerns regarding excessive stress and potential adverse health effects resulting from an extended subchronic oral gavage study motivated a change in the method of toxicant exposure from oral gavage to a feeding study. Data from the 14-day gavage study estimated daily doses (nominal) of 0, 50, 100, 500, 900, and 1400 mg 2A-DNT/kg-day body weight for a subchronic 40-day feeding experiment. An additional

noncontaminant exposed group was included that consisted of 10 each male and female mice (Feed Restricted (FR)) that had their ration reduced equivalent to food intake by the 1400 mg/kg-day dose group. The assumption was that mice in the highest dose group would exhibit varying levels of aversion to 2A-DNT-contaminated food and that including an FR group would help to differentiate effects due to contaminant exposure and those resulting from a reduced nutrient intake. Aversion to contaminated food items can provide survival and protective benefits for wild species under natural conditions.

(b) At day 40, there was no difference in change of body weight or food intake in male or female mice; there were no interactive effects with sex and dose (Figures 12 and 13). There was notable food aversion for the first 7 to 14 days in males and females in the 1400 mg/kg-day group when compared to their pretreatment intake. This resulted in a maximum decrease (day 7) of 10.2 and 10.5 percent body weight in males and females, respectively. Males, and to a lesser degree in females, increased their food intake with body weights closely approaching pretreatment levels by the end of the 40-day experiment. A reduction in food availability, along with a commensurate drop and a gradual recovery in body weight in male and female in the 1400 mg/kg-day dose group was similar to the same pattern exhibited in the FR mice, which suggests changes in body mass was a result of food avoidance than toxicity.

(c) The hematology endpoints RBC count and Hgb concentration showed treatment related decreases beginning at 900 and 500 mg/kg-day, respectively (see Figure 14). Hematocrit (packed cell volume or PCV) was decreased only at 900 mg/kg-day compared to the FR group (data not shown). There were no plasma chemistry differences. Absolute liver, kidney, and spleen weights showed variable enlargement in male and female mice exposed to dietary 2A-DNT (see Figures 15 and 16). Males showed greater sensitivity in an increase in mass of the liver, kidney, and spleen. These three organs were significantly larger (liver – 36 percent, kidney – 20 percent, spleen – 211 percent) at 500 and 900 mg/kg-day in males, whereas a similar enlargement (liver – 26 percent, spleen – 209 percent) in females was observed beginning at 900 mg/kg-day. Histopathology results for these tissues are currently pending.

(d) The only notable clinical signs of 2A-DNT exposure observed in the subchronic study were chromaturia, lethargy, a slight hunched posture, and an apparent stiffness in gait with movement. Mice in the FR group exhibited no adverse changes in the measured endpoints with values no different from those of the control group. At the end of the experiment, survival was 90 percent in males and slightly higher in females at 91.4 percent, with most deaths in the high exposure group.

(e) Based on decreased Hgb concentration and an increased mass of the liver

and spleen, these data initially suggest a LOAEL of 500 mg/kg-day and a NOAEL of 100 mg/kg-day. When daily measured food consumption data for each dose group was used in the determination of effect levels along with the average body weight per group, the LOAEL value becomes 757 mg/kg-day and the NOAEL value becomes 169 mg/kg-day.

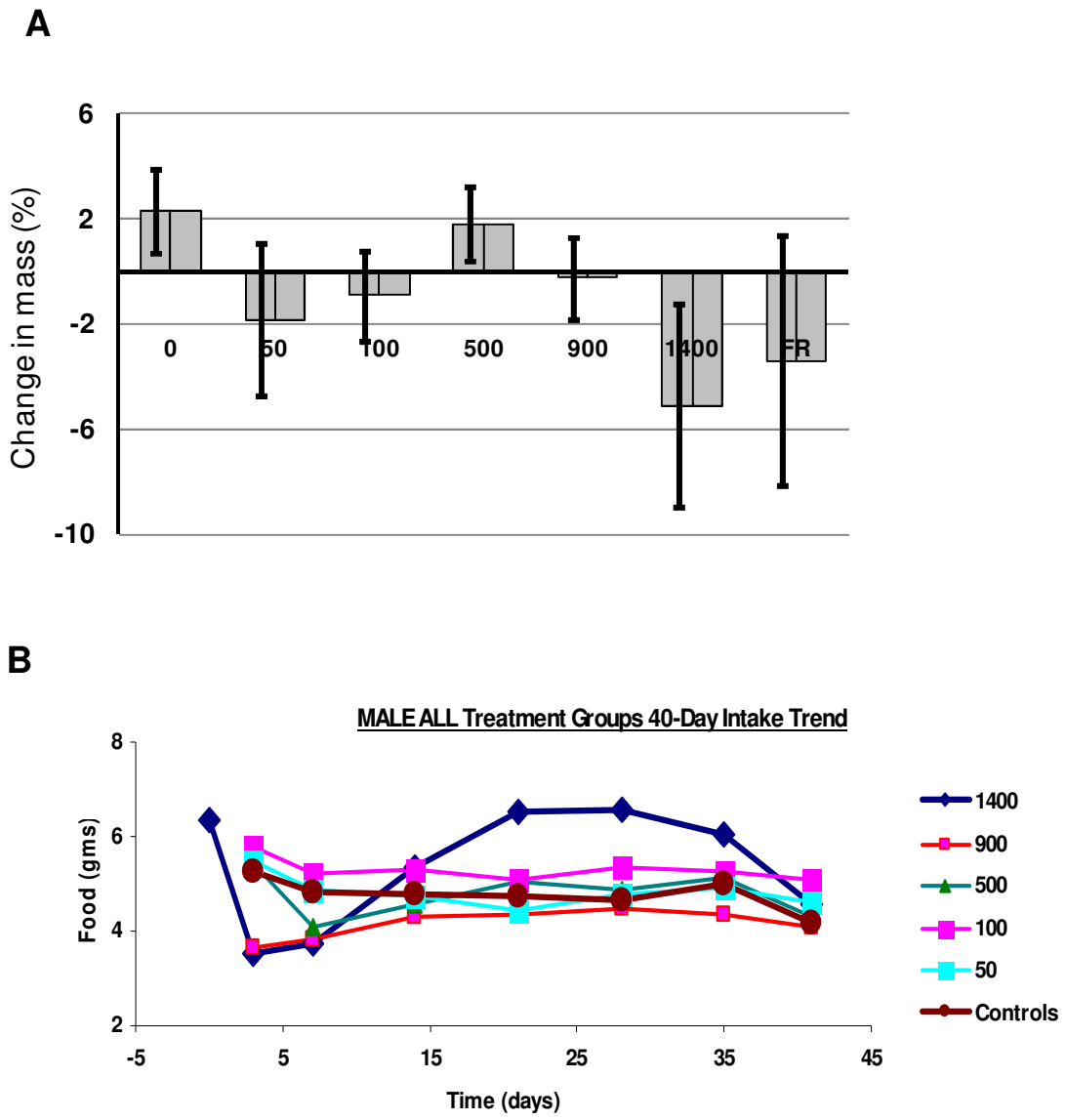

Figure 12. Changes in Mean Male Body Mass (A) and Feed Consumption (B) in *P. leucopus* Relative to Food Concentrations of 2A-DNT Exposed for 40 Days

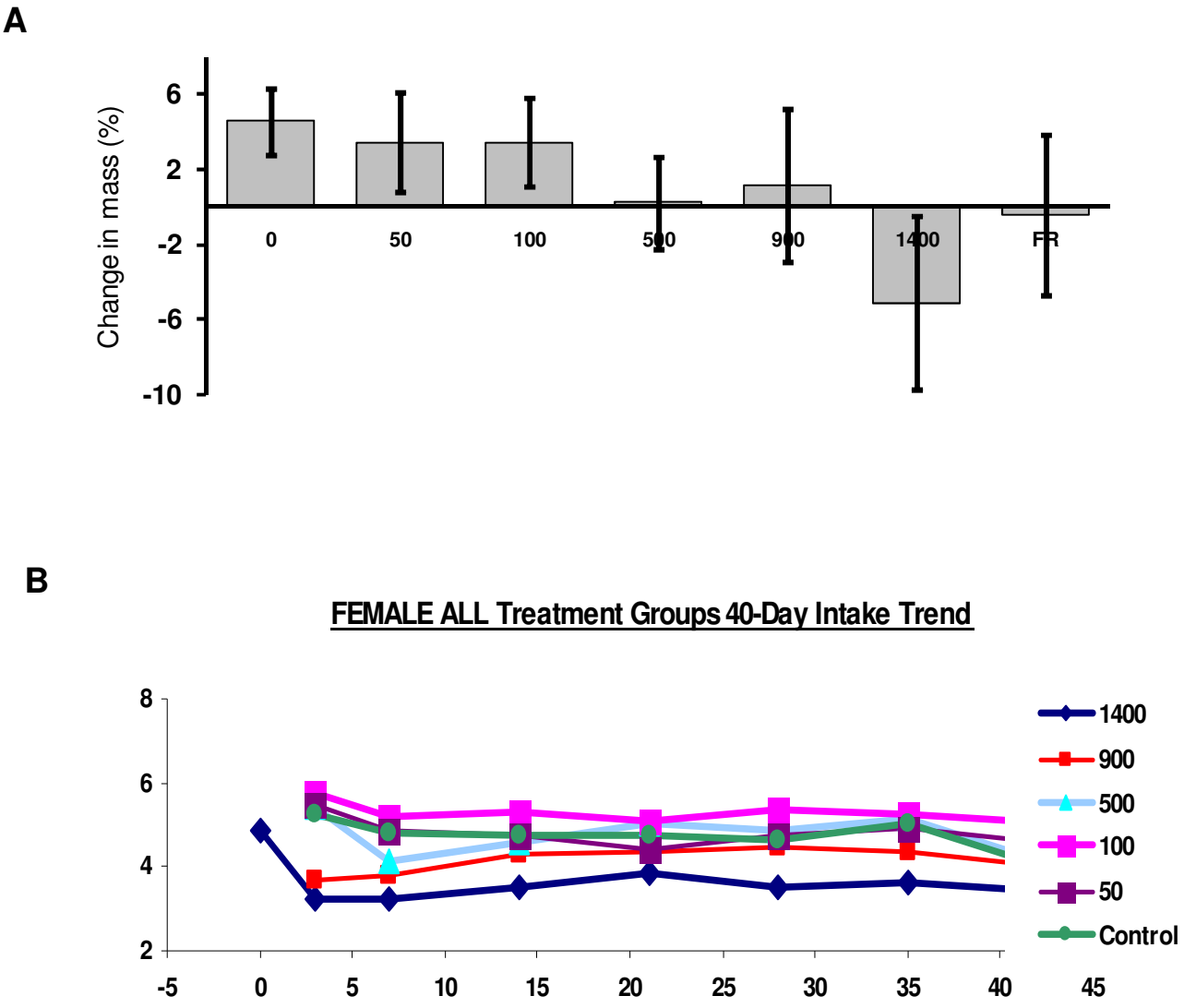

Figure 13. Changes in Mean Female Body Mass (A) and Feed Consumption (B) in *P. leucopus* Relative to Food Concentrations of 2A-DNT exposed for 40 Days

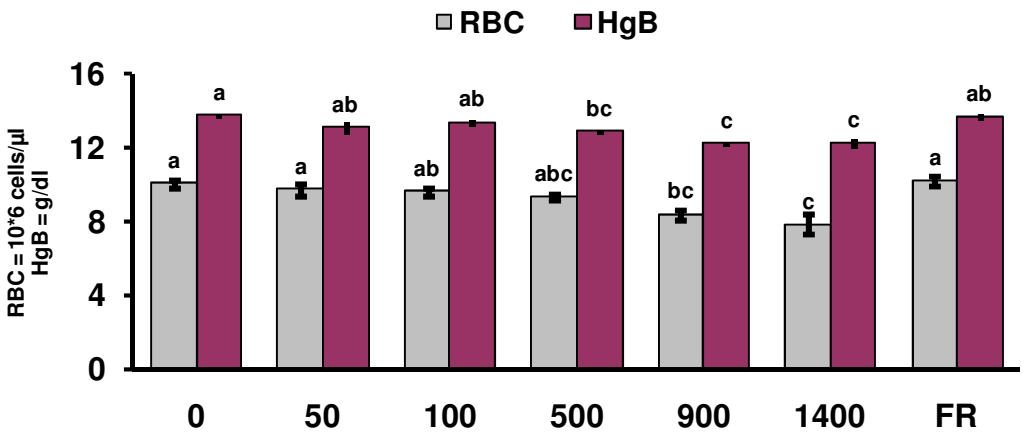

Figure 14. Changes in Mean RBC and Hgb in *P. leucopus* Relative to Food Concentrations of 2A-DNT Exposed for 40 Days
(Bars with different superscripts are different at $p < 0.05$).

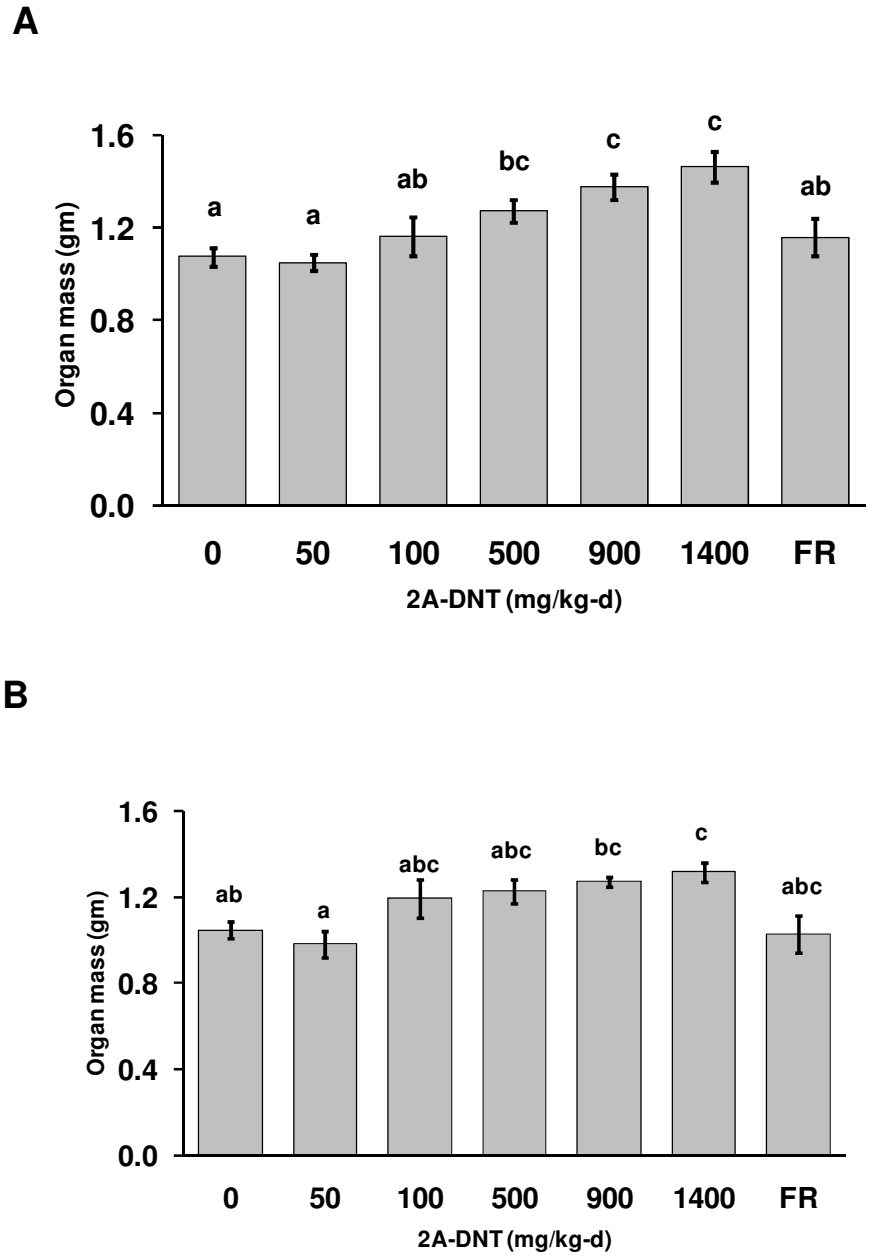

Figure 15. Mean Male (A) and Female (B) Liver Mass in *P. leucopus* Relative to Food Concentrations of 2A-DNT Exposed for 40 Days
(Bars with different superscripts are different at $p < 0.05$).

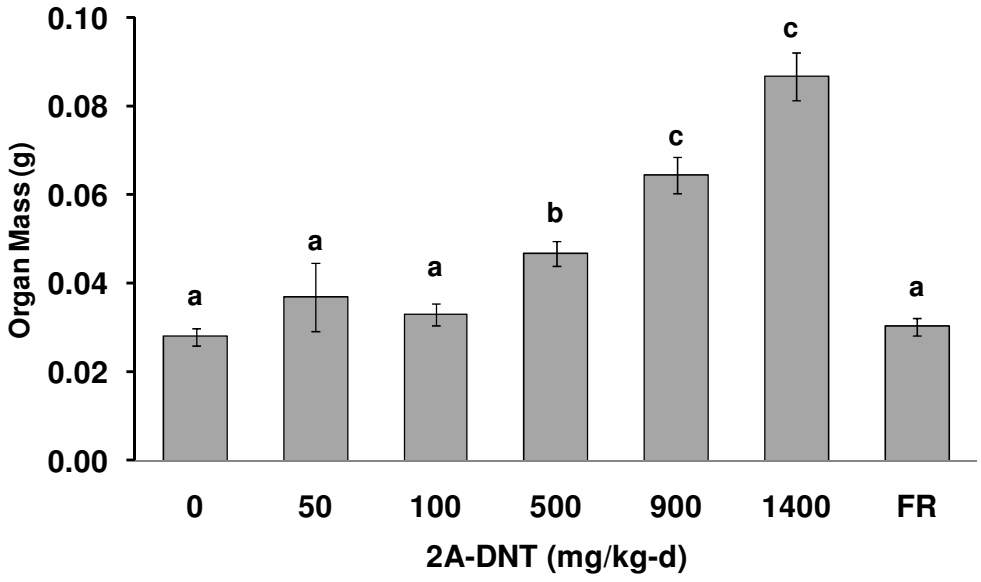

Figure 16. Changes in Mean Spleen Mass in male and female (sexes combined) *P. leucopus* Relative to Food Concentrations of 2A-DNT Exposed for 40 Days (Bars with different superscripts are different at $p < 0.05$).

j. Reptile A-DNT Study.

(1) Acute Study. The first sign of exposure, frequently noted within 6 hours of dosing, was a light yellow to orange discoloration of the area surrounding the vent which resembled parent compound and was different from the observed chromaturia described from studies using TNT in the same species (McFarland et al. 2008). At the lower 2A-DNT doses, lizards remained alert and responsive, with lethargy and minimal escape/avoidance behavior observed in the higher exposures. With doses equal to or above 1360 mg/kg, lizards often became extremely depressed and were unable to right themselves. Some of these high dose lizards were ataxic and appeared disoriented. Tremors and muscular fasciculations were seen in approximately 30 to 40 percent; most of these died 3 to 5 days after being dosed. Survivors to 14 days typically showed signs of improvement at day 5. Lizards lost an average of 12.4 percent body weight across all dose groups. As a general rule, males showed more severe and exaggerated signs of toxicity than females. Probit analysis of the mortality data gave an estimated LD_{50} value (95 percent CI) of 1406 (947-2087 CI) and 1867 (1076-3237 CI) mg/kg body weight for male and female lizards, respectively. The probit/log(dose) slope was 3.885 for males and 2.583 for females. Kaplan-Meier log-rank analysis determined survival times of 9.3 days for males and 9.9 days for females. There was no difference in the median lethal dose ($p > 0.05$) or survival time ($p = 0.826$) between male and female lizards.

(2) Subacute Study.

(a) Significant effects on survival were observed in the 95 and 150 mg/kg-day groups ($p < 0.001$). Mean survival at these doses was 6.8 and 5.8 days, respectively; whereas, average survival time in the five lower groups (0–60 mg/kg-day) was significantly higher at 13.6 days. Survival to less than 14 days in all dose groups prevented a more comprehensive analysis of measured health endpoints. Twenty-eight of forty-two lizards (67 percent) dosed in the subacute experiment survived until scheduled necropsy. Mean daily consumption of crickets with 2A-DNT decreased significantly at 24 to 38 mg/kg-day (3.17 to 1.58) and remained below 1.37 in the higher dose groups ($p < 0.001$). A significant positive dose-response relationship was observed between 2A-DNT dose and a percent loss of body mass in the 0 to 60 mg/kg-day ($p < 0.0001$). Lizards in the 95 and 150 mg/kg-day groups lost considerably less weight than at the lower doses due to shorter survival periods and less time to experience as much weight loss.

(b) Subtle signs of toxicity were noted starting on day 2 and included lethargy and partial to complete anorexia. All lizards dosed with at least 60 mg/kg-day showed depressive effects, while lizards in the 95 and 150 mg/kg-day groups exhibited more exaggerated signs and were markedly toxic (e.g., weak, inactive, and vomiting) at 48 to

72 hours. Within 5 to 7 days, lizards in the 95 and 150 mg/kg-day groups became moribund. At the same time as the clinical signs, a moderate yellow to orange chromaturia and feces with staining around the vent was seen in many lizards, again, primarily in the two high groups. Blood and plasma chemistries were nonsignificant across the exposure groups.

(3) Subchronic Study.

(a) Overt Toxicity.

i. Overall animal survival decreased from 67 to 42 percent despite a reduction in the average daily exposure level from 64 mg/kg in the subacute study to 19 mg/kg in the subchronic study. A significant difference in the number of survival days ($p < 0.0001$) and surviving individuals (χ^2; $p < 0.01$) was noted when the 2A-DNT dose increased from 5 to 15 mg/kg-day (Figure 17). After an average exposure period of 54 days at 15 mg/kg-day, cumulative 2A-DNT dose exceeded 800 mg/kg along with a progressive increase in lethality in the remaining dose groups. No lizards survived to 60 days in the 25 and 30 mg/kg-day groups. Signs of toxicity were similar to those in the subacute study, though more subtle, and are best characterized by anorexia and generalized, progressive cachexia (a wasting syndrome characterized by loss of weight and weakness). A decrease in appetite and activity level, along with a characteristic yellow-orange discoloration around the vent, was noticed within 5 to 7 days after beginning dosing.

ii. Daily consumption of crickets and loss of body weight (Figure 18) was generally well correlated with increasing doses of 2A-DNT. Food intake was significantly reduced compared to the controls in the 15 to 30 mg/kg-day groups ($p < 0.001$). While lizards in the control and 5 mg/kg-day groups showed a net gain in body weight over the 60-day exposure period, lizards in the 15 to 30 mg/kg-day groups showed a loss of between 10.4 to almost 16 percent of their initial body weights ($p < 0.001$).

iii. As noted in the earlier acute and subacute stages, a yellow-to-orange chromaturia and staining of the skin around the vent was observed in the subchronic experiment in 92 percent of 2A-DNT treated lizards. Staining of the vent area was apparent in approximately 25 percent of the dosing period in the 5 mg/kg-day group and 50 percent of the dosing period in the 15 to 30 mg/kg-day groups. Chromaturia was observed 0, 25, 51, 48, 57, and 55 percent of the animals during the dosing period in the control, 5, 15, 20, 25, and 30 mg/kg-day groups, respectively. A threshold was seen between the 5 and 15 mg/kg-day dose groups where the probability of observing chromaturia in treated lizards approximately doubled (Figure 19). The probability of observing chromaturia during the subchronic experiment was significantly greater in

lizards in dose groups receiving in excess of 15 mg/kg-day ($p < 0.05$).

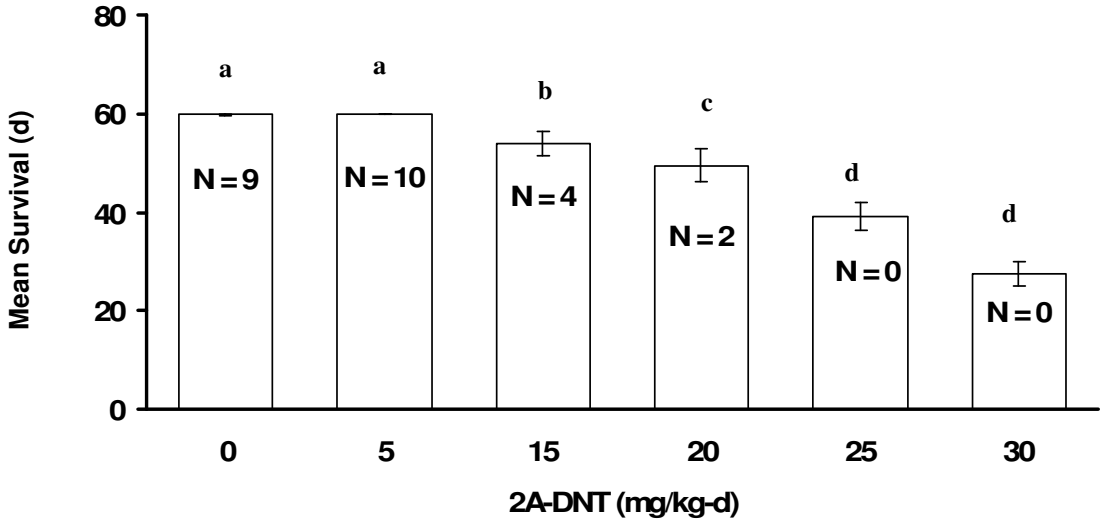

Figure 17. Mean Survival in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days
(N = number of lizards surviving to 60 days; bars are SEM; means with different lower case letters are different at $p < 0.05$).

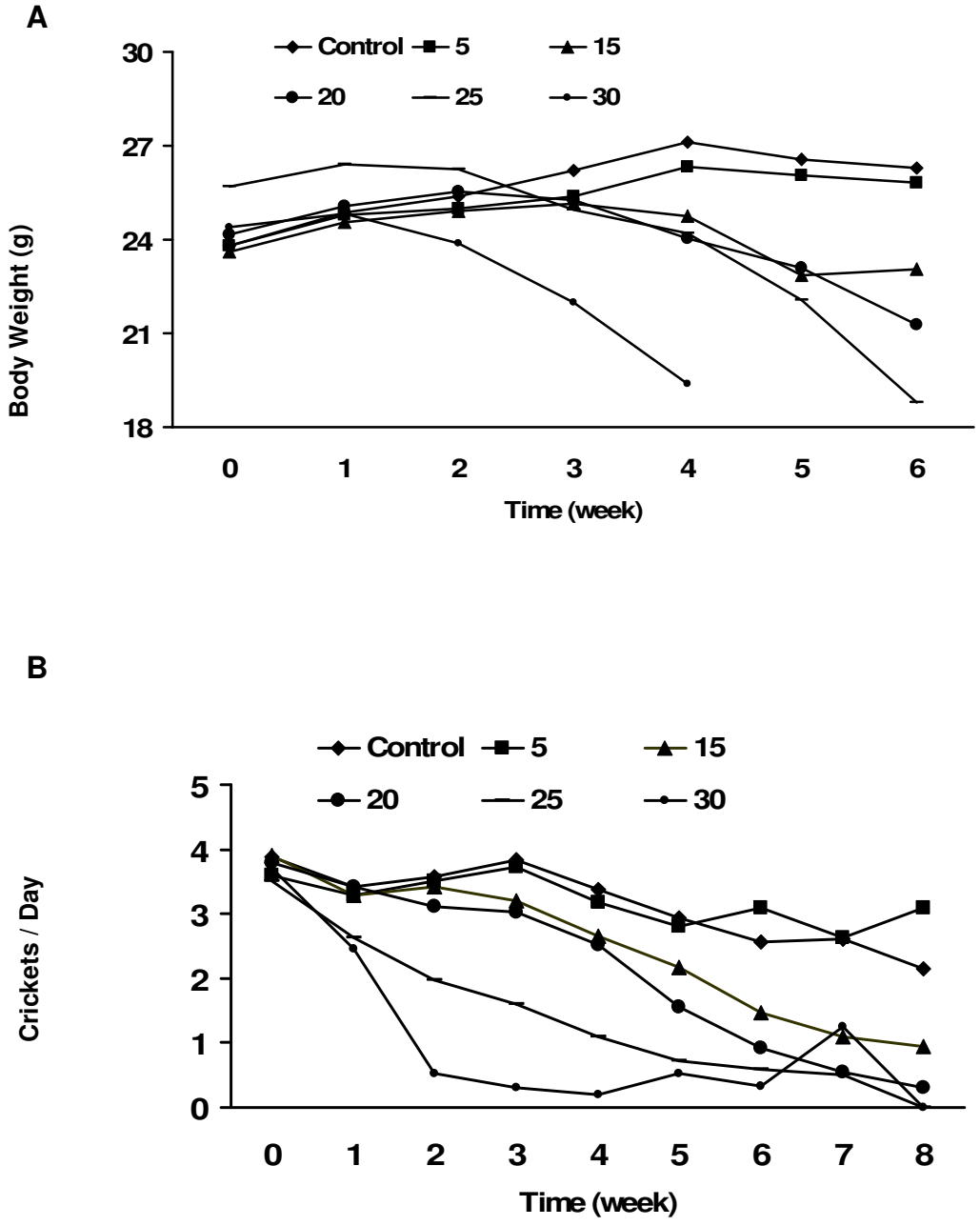

Figure 18. Change in Body Weight (A) and Consumption of Crickets (B) Over Time in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days

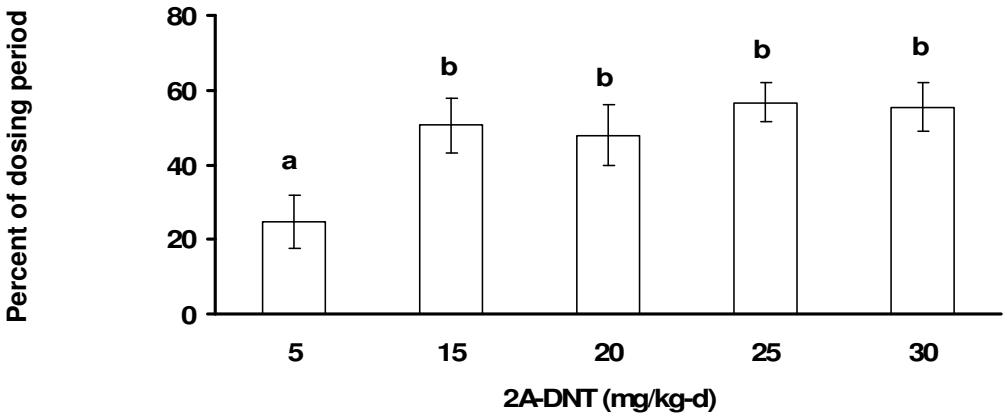

Figure 19. Percentage of the 60-Day Dosing Period When Chromaturia was Observed in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT (Bars are SEM; means with different lower case letters are different at $p < 0.05$).

(b) Organ Weights. Due to the overall variability and significant losses of body weight in the higher dose groups, absolute organ weights were obtained and compared over the range of exposure groups. Though absolute liver weights exhibited no differences from exposure to 2A-DNT ($p = 0.46$), kidney weights were significantly different at 15 and 30 mg/kg-day ($p < 0.001$; Figure 20). Kidney mass in the 15 to 30 mg/kg-day dose groups was between 41 and 94 percent greater compared to controls. Despite the absence of a clear dose-dependent relationship, significant splenic enlargement was seen in the 25 mg/kg-day group ($p < 0.001$), along with an increase in mass (vs. controls) of 36 to 80 percent in the four high-dose groups (Figure 21). No differences were found when comparing brain weight and 2A-DNT dose ($p = 0.059$). Gross testicular atrophy was observed in animals in the 15 to 30 mg/kg-day groups with an absolute testes mass of 58 to 24 percent of the controls ($p < 001$; Figure 22).

(c) Histopathology.

i. In the kidney, minimal to mild atrophy of the renal tubules characterized by loss of the bright staining apical eosinophilic cytoplasm and reduced epithelial height was observed in lizards given 15 mg/kg-day. Multifocal to confluent mild-to-moderate hyperplasia and/or minimal to moderate hypertrophy of renal convoluted tubular epithelium with occasional focal epithelial atypia was noted in lizards given 15 and 20 mg/kg-day. The frequency of renal tubular changes was dose-dependent.

The incidence of hyperplasia was significantly different from controls in the 15 (7/10), 20 (6/8), 25 (8/9), and 30 (7/9) mg/kg-day groups, and different from controls regarding hypertrophic changes in the 15 (8/10), 20 (6/8), 25 (8/9), and 30 (8/9) mg/kg-day groups ($p < 0.05$).

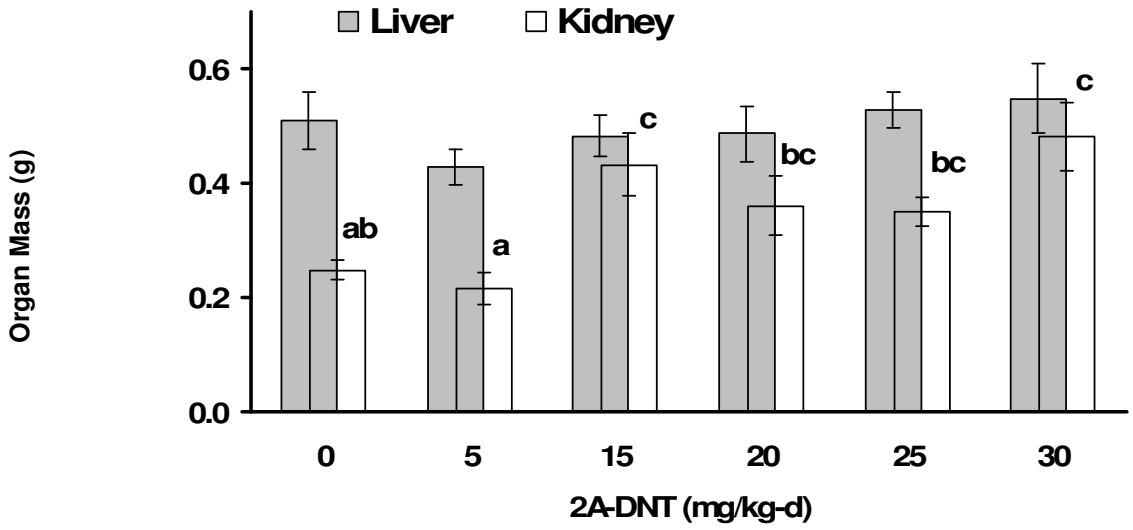

Figure 20. Changes in Absolute Organ Weights in Liver ( ) and Kidney ( ) in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days (Bars are SEM; means with different lower case letters are different at $p < 0.05$).

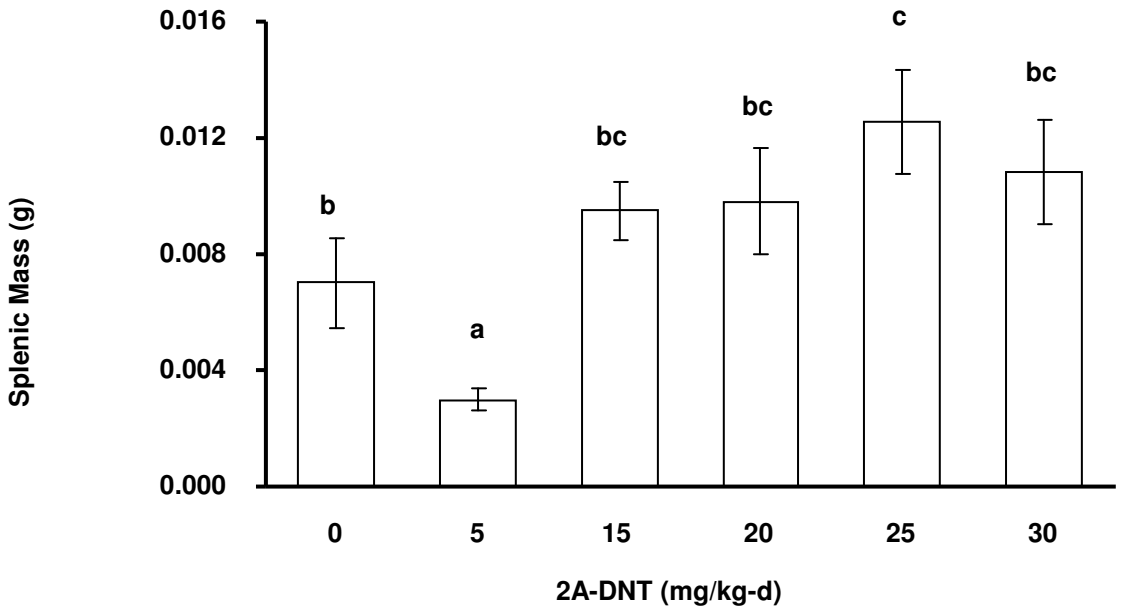

Figure 21. Changes in Absolute Spleen Weights in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days (Bars are SEM; means with different lower case letters are different at $p < 0.05$).

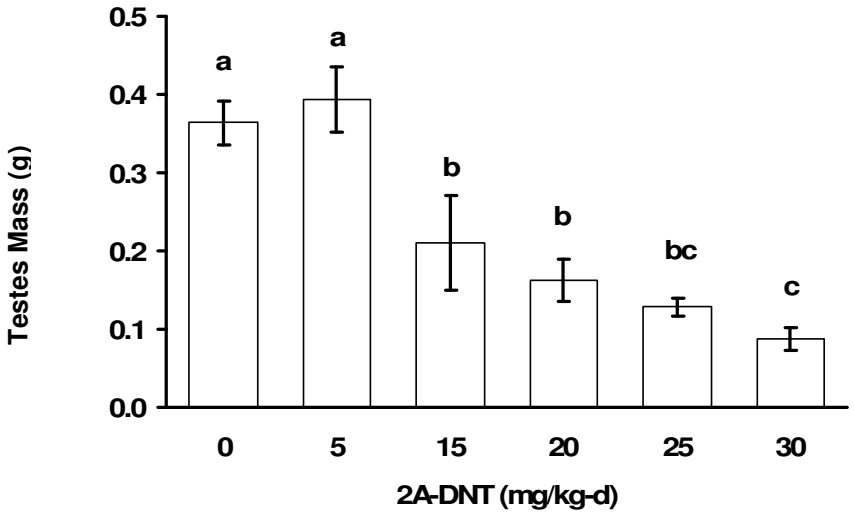

Figure 22. Changes in Absolute Testes Weights in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days (Bars are SEM; means with different lower case letters are different at $p < 0.05$).

ii. A dose-dependent minimal to moderate hepatocellular transdifferentiation was noted in the hepatic tissue of lizards given at least 5 mg/kg-day. Minimal changes were evident as an increase in periportal cellularity composed of cords of spindle-shaped cells extending into the adjacent hepatic parenchyma; this was considered consistent with early biliary hyperplasia. In the more severe cases, changes progressed and transitioned to include hepatocytes that were reduced in size with increased basophilia organized into plate-like structures, ducts, and ascini. Many of these foci resembled immature bile ducts and pancreatic ascini. With increasing severity, changes extended across the hepatic lobule. Since there was no evidence of hepatocellular degeneration or necrosis to suggest a proliferative or reparative response to injury, the hepatic finding was considered most consistent with hepatocellular dedifferentiation and phenotypic transdifferentiation. These hepatic changes were significant in the 15 (7/10), 20 (7/8), 25 (7/9), and 30 (7/9) mg/kg-day groups ($p < 0.05$).

iii. In the spleen, minimal to mild reticuloendothelial cell hyperplasia accompanied by lymphoid depletion was observed in lizards given 5 mg/kg-day or more. Splenic hyperplasia was significant at the 25 (6/9) mg/kg-day dose ($p < 0.05$). In affected lizards, reticuloendothelial cells and histiocytes made up a greater portion of the spleen, while in controls the predominant cell type was lymphocytes.

iv. In testes, bilateral mild seminiferous tubule epithelial atrophy or moderate epithelial degeneration was noted in 2/10 lizards given 15 mg/kg-day. Atrophy was characterized primarily by depletion of elongate and round spermatids and with associated foci of epithelial degeneration. Degeneration, in the absence of atrophy, was characterized by foci of epithelial degeneration in most tubular segments accompanied by nodular histiocytic infiltrates. Significant mild to moderate tubular atrophy was noted in lizards at 30 (5/9) mg/kg-day ($p < 0.05$). In addition, variable and nonsignificant, minimal to mild hyperplasia and/or hypertrophy of the gallbladder and the large intestinal mucosal epithelium were observed at 15 mg/kg-day.

(d) Clinical Chemistry and Hematology.

i. Clinical chemistry and hematology data were available only in lizards necropsied at the scheduled termination of the 60-day experiment (controls, 5, 15, and 20 mg/kg-day). Exposure to 2A-DNT produced no consistent or treatment-related changes in any of the plasma chemistry parameters that were evaluated in the Western fence lizard except for subtle increases in BUN, CREA, and URIC (Table 16). While trends in these parameters appear dose-related, only BUN values for lizards in the 15 mg/kg-day group exhibited significant increases ($p < 0.05$). The single individual lizard value at 20 mg/kg-day for BUN, CREA, and URIC was greater than the measurements at 15 mg/kg-day.

ii. Hematologic analyses did not reveal any significant differences between control lizards and those exposed to 2A-DNT except for a decrease in HCT at 5 mg/kg-day ($p < 0.05$). Hemoglobin, RBC count, and TS showed nonsignificant decreases over the measured dose range (Table 17). The WBC count, as well as the indices mean corpuscular volume (MCV) and mean cellular hemoglobin content (MCHC), were highly variable and without relationship to 2A-DNT dose.

(e) Spermatozoan Analysis. Total spermatozoan counts and motility measures in all treatments were variable and nonsignificant ($p = 0.514$, data not shown). Epididymal and vas deferens spermatozoan counts, motility, and progression exhibited a decreasing trend in the control, 5, and 15 mg/kg-day groups, with values between 42 and 80 percent of the controls in the two lowest-dosed groups.

Table 16. Blood Chemistry in Male Western Fence Lizards (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days (Data are mean ± SEM; sample size in parentheses; *indicates different from control mean at $p < 0.05$)

		2A-DNT (mg/kg-d)			
Parameters		0 (n)	5 (n)	15 (n)	20 (n)
ALB (g/dL)		2.01 ± 0.20 (8)	1.64 ± 0.23 (8)	1.40 ± 0.18 (4)	1.60 (1)
ALKP (U/L)		34.00 ± 4.04 (6)	25.57 ± 5.38 (7)	34.67 ± 10.18 (3)	45.00 (1)
AST (U/L)		104.17 ± 11.42 (6)	64.14 ± 11.67 (7)	80.67 ± 19.72 (3)	121.00 (1)
BUN (mg/dL)		2.67 ± 0.21 (6)	2.86 ± 0.14 (7)	3.75 ± 0.48* (4)	6.00 (1)
CREA (mg/dL)		0.53 ± 0.08 (6)	0.64 ± 0.09 (7)	0.73 ± 0.29 (3)	1.10 (1)
Phosphorus (mg/dL)		10.26 ± 0.64 (7)	8.45 ± 0.47 (8)	13.83 ± 2.88 (3)	11.80 (1)
URIC (mg/dL)		1.31 ± 0.26 (7)	1.33 ± 0.25 (8)	1.90 ± 0.99 (4)	2.00 (1)

Table 17. Hematology Parameters in Male Western Fence Lizards (*Sceloporus occidentalis*) Dosed Orally with 2A-DNT for 60 Days (Data are mean ± SEM; sample size in parentheses; *indicates different from control mean at $p < 0.05$.

		2A-DNT (mg/kg-d)			
Parameters		0 (n)	5 (n)	15 (n)	20 (n)
Hgb (g/dL)		8.83 ± 0.51 (9)	7.54 ± 0.39 (10)	7.55 ± 0.52 (4)	10.95 ± 2.86 (2)
Hct (%)		32.44 ± 2.22 (9)	26.44 ± 1.66* (10)	28.25 ± 1.93 (4)	38.50 ± 8.53 (2)
RBC (10^6/µL)		1.52 ± 0.12 (9)	1.24 ± 0.07 (10)	1.18 ± 0.06 (4)	1.22 (1)
TS (g/dL)		4.61 ± 0.19 (9)	4.51 ± 0.27 (10)	4.35 ± 0.17 (4)	5.00 ± 0.80 (2)
WBC (10^3/µL)		121.50 ± 11.22 (9)	94.70 ± 6.34 (10)	139.38 ± 24.10 (4)	158.50 (1)
MCV ((fl)		216.67 ± 8.36 (9)	211.05 ± 7.11 (10)	239.27 ± 10.02 (4)	245.90 (1)
MCHC (g/dL)		27.43 ± 0.59 (9)	29.05 ± 0.56 (10)	26.74 ± 0.41 (4)	28.18 ± 1.18 (2)

k. Reptile RDX Study.

(1) Acute Study. Indications of central nervous system neurotoxicity were observed within 12 hours of dosing and included depression/hyperactivity, tremors, ataxia and paresis (partial loss of or impaired movement), and intermittent tonic-clonic generalized seizures (ictus), as well as anorexia and weight loss. Initially, animals would often exhibit body rigidity and later become inactive and nonresponsive to handling. Depression was exhibited by lizards that were nonresponsive or poorly responsive to normal auditory and/or tactile (handling) stimuli; whereas normal behavior was observed in animals which were much more readily aroused by similar stimuli. Lizards that were able to live 60 hours survived to the end of the 14-day observation period. Twelve of forty-four lizards (27 percent) exhibited seizure activity (9 males, 3 females), with four of the six individuals that received the lowest seizure dose (75 mg/kg) considerably improved at 72 to 96 hours and able to survive the 14-day observation period. The other eight lizards with seizures were in the higher dose groups (95, 120, and 135 mg/kg) and died, or were humanely euthanized, at 18 to 24 hours. Probit analysis estimated oral LD_{50} values (95 percent CI) as 72 (49-106 CI) and 88 (65-119 CI) mg/kg body weight for male and female lizards, respectively; probit/log (dose) slope was 3.8 for males and 4.5 for females. Kaplan-Meier log-rank analysis calculated survival times of 6.5 days for males and 7.5 days for females. There was no difference in LD_{50} ($p > 0.05$) or survival time (log-rank test statistic, $Z = 0.156$; degrees of freedom, $df = 1$; $p = 0.693$) between males and females.

(2) Subacute Study. A significant dose-survival relationship was seen over the range of exposures in the subacute study ($H = 35.64$; $df = 6$; $p < 0.001$); no lizards that received at least 20 mg/kg-day survived to the completion of the subacute study, limiting many analyses to the control and 10 mg/kg-day dose groups. An apparent threshold occurred when mean survival decreased from 14 to 7.3 days between the 10 and 20 mg/kg-day dose groups. Average survival time (as a percent of 14 days) for the control, 10, 20, 25, 30, 45, and 60 mg/kg-day groups was 100, 100, 52.4, 35.7, 23.8, 13.1, and 9.5 percent, respectively; observed variation in the mean survival time for the same dose groups was 14, 14, 7.3, 5.0, 3.3, 1.8, and 1.3 days, respectively. As was observed with the acute experiment, RDX-dosed lizards also exhibited anorexia and weight loss, inactivity, paresis, tremors, and tonic-clonic seizures. Depression, seizure activity precipitated as a result of handling, flaccid paralysis, and death were seen within 24 hours of dosing at the beginning of the experiment. Lizards in the 10 and 20 mg/kg-day groups most commonly displayed inactivity, weakness, and depression, while signs observed in the higher-dosed individuals suggested more severe neurologic dysfunction (e.g., seizures, postictal flaccid paralysis, and paddling-running motions). Seizure activity was observed in lizards from all RDX-dosed groups in the subacute study except in the 10 mg/kg-day group. Subcutaneous muscular fasciculations were often palpated when handling lizards with seizures. Lizards in the subacute study were

often observed displaying a characteristic and easily recognizable pattern of abnormal body postures (i.e., upward extension of the tail, back arched dorsally, and forelimbs extended posteriorly and pressed against the body wall). Similar body postures were also noticed during the acute phase of the study but were usually exhibited separately and consisted mostly of generalized body rigidity and/or extension of the tail when handled. Cricket consumption was markedly reduced in a dose-related manner to near zero at the high dose group, with only the control lizards displaying a normal intake of crickets. Data sufficient for an analysis of change in body weight was limited to four treatment groups (0, 10, 20, and 25 mg/kg-day), where no significant changes were observed. Blood and plasma chemistry analyses in the subacute study were unremarkable and without significant differences among the treatment groups (data not shown).

(3) Subchronic Study.

(a) Compared to the subacute experiment, percent survival increased nearly two-fold in the subchronic study when the total dose of RDX administered was reduced by approximately 38 percent. Thirty-four (57 percent) of the sixty lizards survived until the scheduled necropsy at the end of the subchronic experiment (Figure 23). Twenty-four deaths occurred in the 5, 8, and 11 mg/kg-day groups with a significant and dose-dependent decrease in the 8 and 11 mg/kg-day groups ($H = 43.08$; $df = 5$; $p < 0.001$); poor survival in the higher two dose groups limited sample size and compromised many statistical comparisons. Early in the study, and especially in the lower dose groups, most lizards appeared alert and were responsive when handled. The most common signs of toxicity observed prior to death, or early euthanasia, were inactivity, decreased responsiveness, appetite reduction, and weight loss; early euthanasia was necessary usually due to significant loss of body mass. One lizard (32 days and 2.5 mg/kg-day dose group) in the 60-day study was observed having seizures. As in the acute and subacute experiments, characteristic and abnormal body postures (often initiated with handling) were observed in approximately 33 percent of the lizards. Some individuals exhibited abdominal enlargement and an accumulation of dried feces surrounding the vent. Lizards that completed the study showed dose-related effects in food intake (crickets) and body weight (Figure 24). Compared to controls, food intake was significantly reduced in the 5, 8, and 11 mg/kg/day groups ($H = 45.35$; $df = 5$; $p < 0.001$), with a progressive loss of body weight over the dose range, significant at the 5, 8, and 11 mg/kg-day doses ($H = 38.90$; $df = 5$; $p < 0.001$; Figure 24).

(b) No significant relationship was found when comparing brain weight and RDX dose ($F = 1.65$; $df = 5, 46$; $p = 0.165$). Liver weights, measured as a ratio of brain weight, exhibited differences as a result of RDX exposure at 8 and 11 mg/kg-day ($F = 4.47$; $df = 5, 46$; $p < 0.005$); livers were 48 and 40 percent larger, respectively, at these two doses when compared to controls (Figure 25). Kidney enlargement was variable and significant at 5, 8, and 11 mg/kg/day but only when compared to the

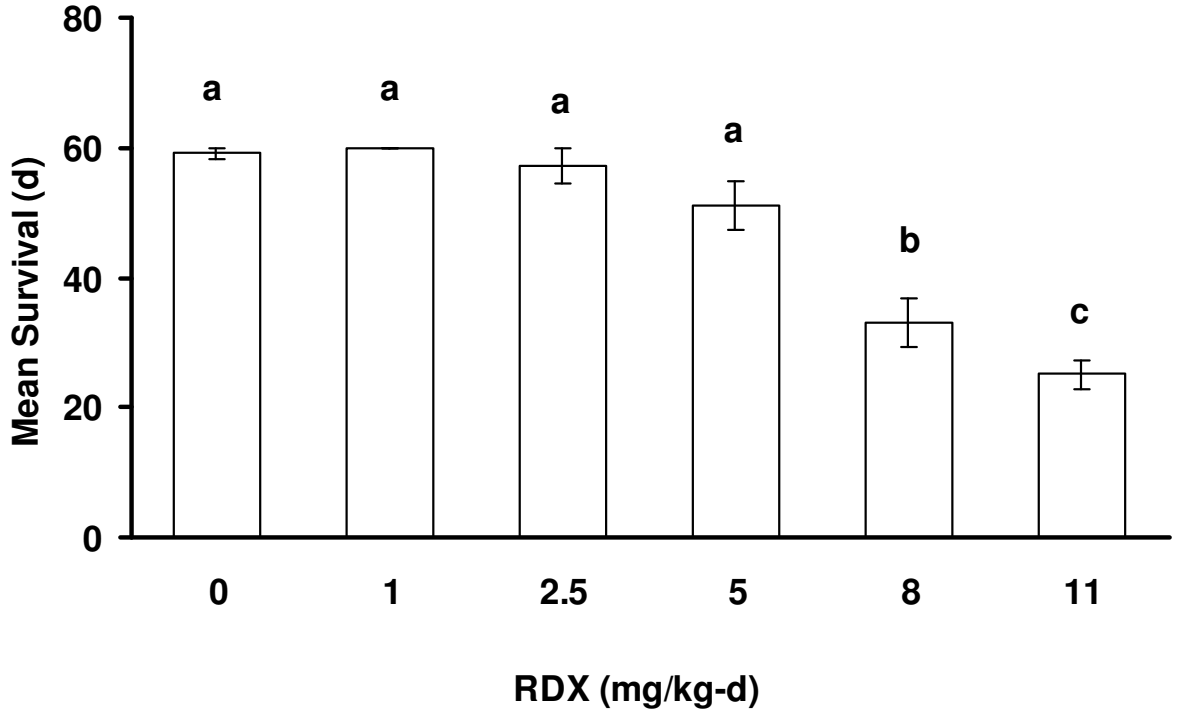

Figure 23. Mean Survival in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with RDX for 60 Days
(Bars are SEM; means with different lowercase letters are different at $p < 0.05$).

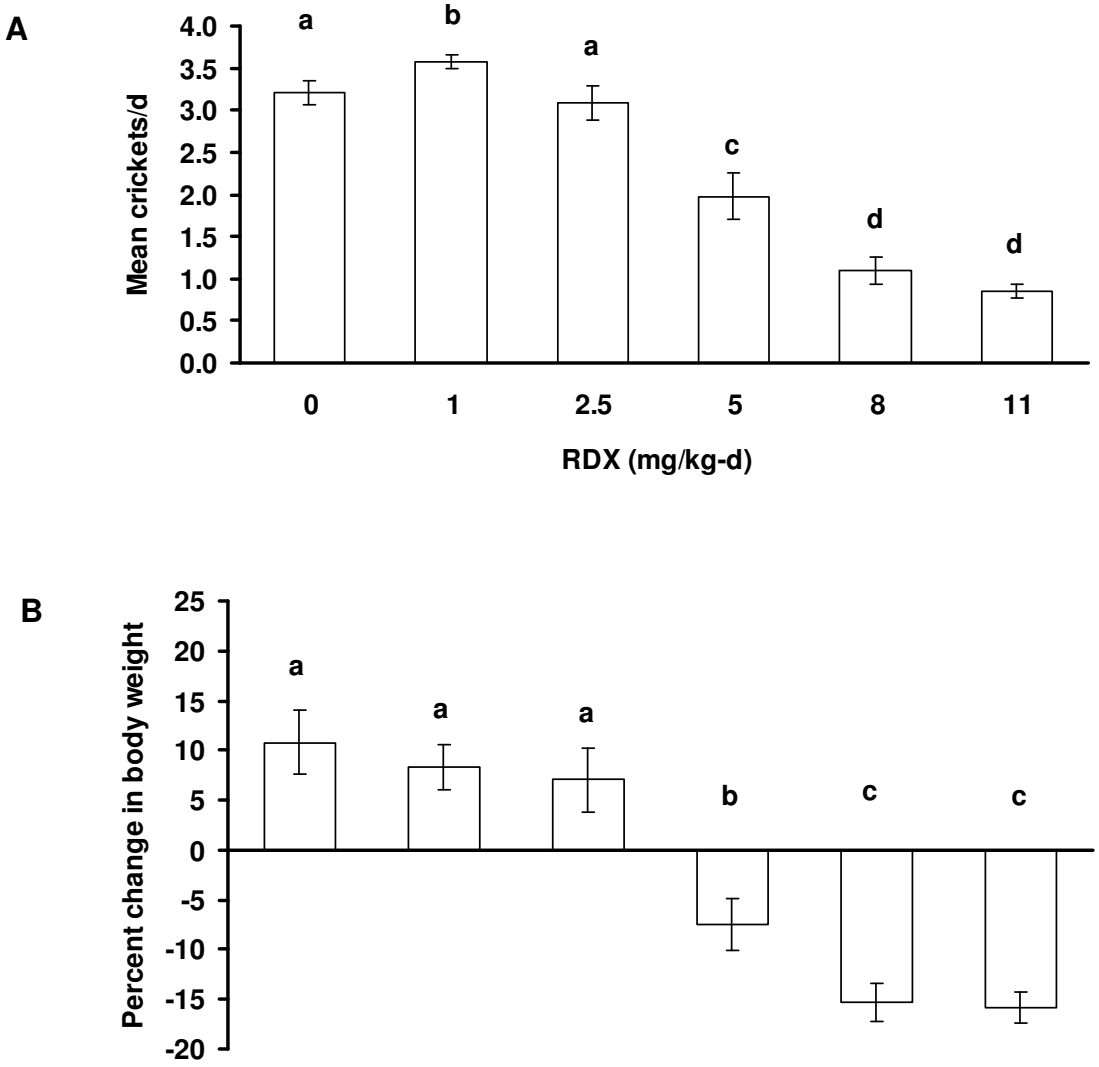

Figure 24. Mean Daily Cricket Consumption (A) and Percentage of Change in Body Weight (B) in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with RDX for 60 Days
(Bars are SEM means with different lowercase letters are different at $p < 0.05$).

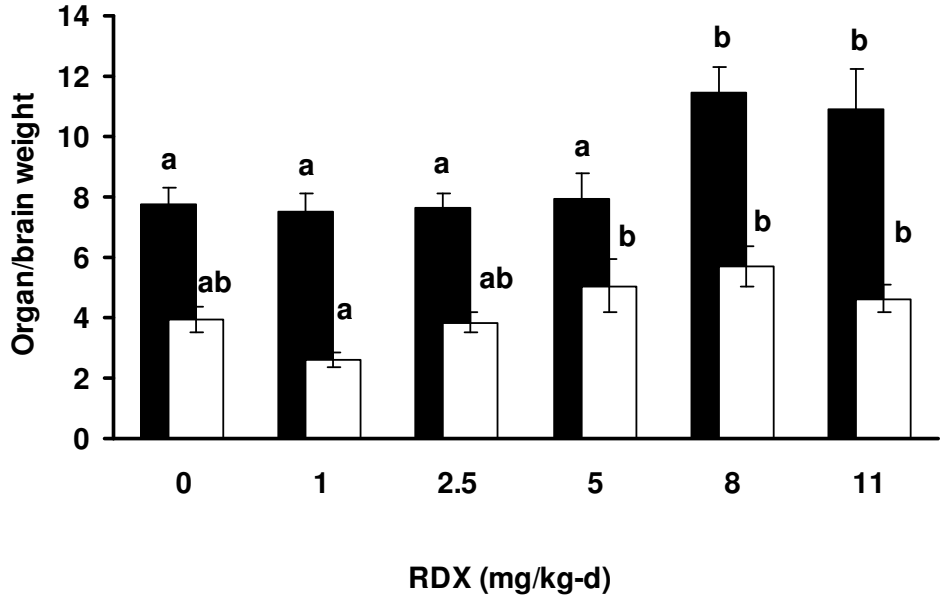

Figure 25. Changes in Relative Organ/Brain Weight Ratios in Liver (■) and Kidney ( ) in the Western Fence Lizard (*Sceloporus occidentalis*) Dosed Orally with RDX for 60 Days
(Bars are SEM; means with different lowercase letters are different at $p < 0.05$).

1 mg/kg-day group ($F = 4.52$; $df = 5, 46$; $p < 0.005$); kidneys were an average of 31 percent larger in the three highest dose groups compared to controls (Figure 25). Changes in spleen:brain weight ratios were highly variable and not related to dose at all exposure levels. Analysis of testes/brain data revealed a significant decrease in the 8 and 11 mg/kg-day groups (38 and 28 percent of control, respectively; $F = 9.29$; $df = 5, 46$, $p < 0.001$). Testes/brain weight exhibited a nonsignificant decreasing trend from 1 to 5 mg/kg/day (see Figure 26).

(c) Renal tubular atrophy and glomerular hyaline droplet change were observed and, in the more severe cases, appeared to progress to proliferative glomerulonephritis in both the moribund lizards and those surviving to 60 days. Bilateral, diffuse seminiferous tubular degeneration, and abnormal spermatogenesis were characterized by degeneration and depletion of elongate spermatids along with necrosis and sloughing of the tubular epithelium. Vacuoles were observed in both intestine and liver and were consistent with lipid deposition. Hepatic vacuoles were predominately minimal with some mild changes in all dose groups. The significance of hepatic

vacuoles is unclear given the presence of the lesion in control group lizards. Splenic white pulp macrophage hyperplasia was seen as an increased size of the macrophage aggregates. Examination of brain revealed tissues within normal limits and no RDX-related histological changes.

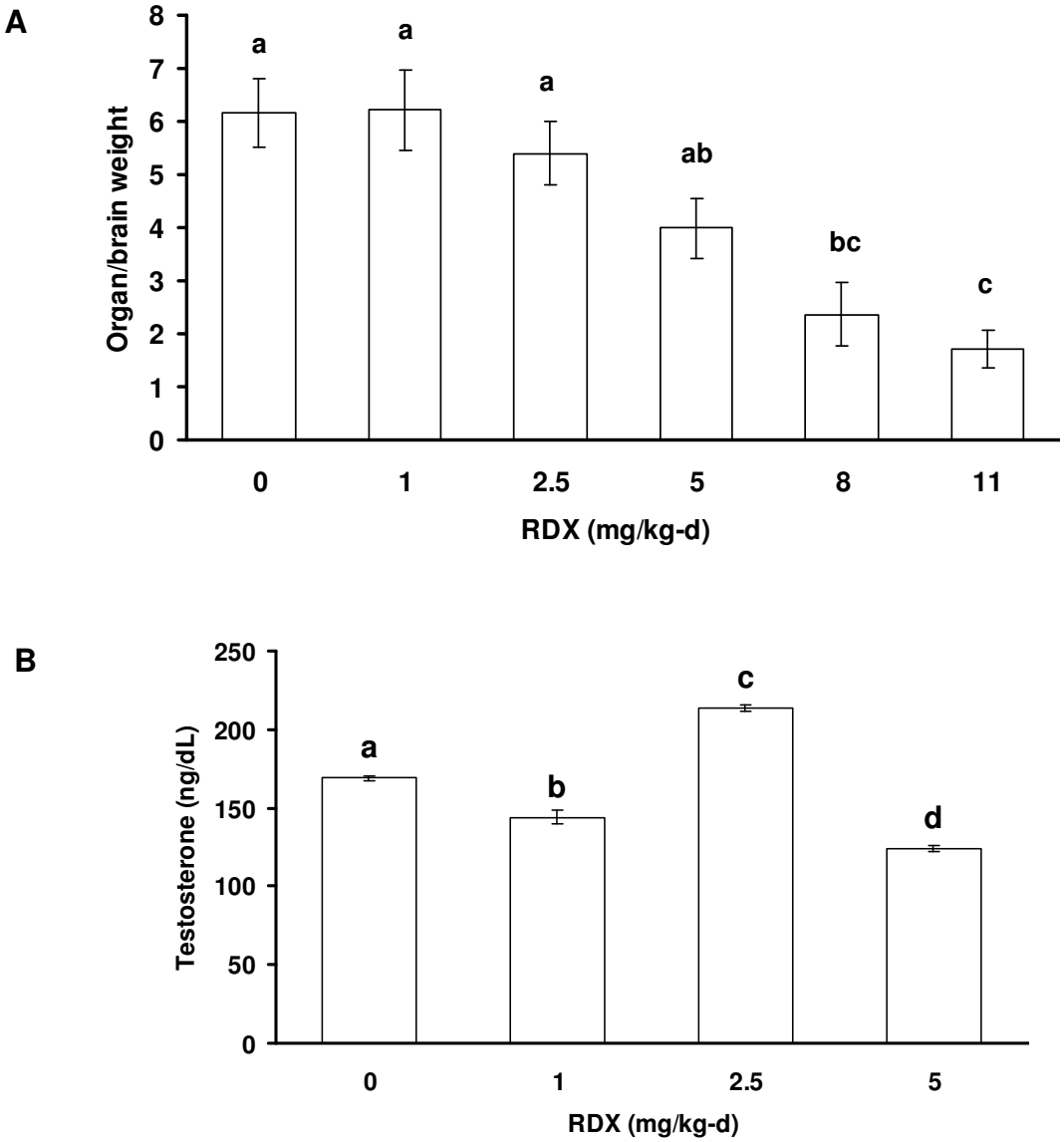

Figure 26. Changes in Relative Testes/Brain Weight Ratios (A) and Plasma Testosterone (B) as a Result of Exposure to RDX for 60 Days in the Western Fence Lizard (*Sceloporus occidentalis*)
(Bars are SEM; means with different lowercase letters are different at $p < 0.05$).

(d) Hematology and plasma chemistry data were available only in lizards surviving to the scheduled end of the experiment (controls, 1, 2.5, and 5 mg/kg-day). Hematologic analyses did not reveal any significant differences between control lizards and those exposed to RDX. Clinical chemistry exhibited no clear dose-related differences, except a decrease in TP, ALB, and GLOB and Ca concentrations in the 5-mg/kg-day group. Most plasma chemistry results were highly variable and with minimal effects only in the 5-mg/kg-day group. Mean values for ALP, CHOL, GLU, lactate, phosphorus, and URIC were lower than controls for lizards in the 5 mg/kg-day group, though they did not follow a dose-response pattern.

(e) Total spermatozoan counts and motility measures were variable for all treatment levels. Epididymal and vas deferens spermatozoan counts were not different across treatments ($F = 1.35$; $df = 4, 29$; $p = 0.274$, data not shown), with no relationship to dose in the 1, 2.5, and 5 mg/kg-day groups and counts that ranged from 42 to 78 percent of the controls.

(f) Change in plasma testosterone concentrations was not related to exposure levels of RDX (Figure 26). Though statistical differences were observed in all analyzed dose groups ($F = 218.6$; $df = 3, 8$; $p < 0.001$), the absence of a dose response suggested the changes lacked biological significance.

(g) RDX was found in all exposed lizards' brain tissue that was collected and analyzed for contaminant residues ($n = 10$). Exposure times varied from 1 to 8 days in the 14-day study and 16 to 25 days in the 60-day study, with brain RDX concentrations ranging from 18 to 41 µg/g and from 1.6 to 16 µg/g, respectively (Figure 27). Mean brain RDX concentrations in the 14-day and 60-day studies were 27.75 µg/g and 8.45 µg/g (Student's *t* test, $t = -3.76$; $df = 8$; $p = 0.006$), respectively, while survival times in the 14-day and 60-day studies were 3.4 days and 19.3 days, respectively (Student's *t* test, $t = 7.88$; $df = 8$; $p < 0.001$); differences were significant comparing brain RDX concentrations and survival time. A significant exponential relationship between survival time and RDX dose was observed ($p < 0.0001$, $r = 0.94$), with the suggestion of a threshold brain RDX concentration between 16 and 18 µg/g in the 60- and 14-day studies where mean survival significantly decreases from 19.33 days to 3.25 days (Figure 27). During the in-life portion of the study, none of the six lizards from the subchronic study exhibited seizures, but all four lizards from the subacute study exhibited seizures, with a seizure activity threshold suggested at a brain RDX concentration between 16 and 18 µg/g.

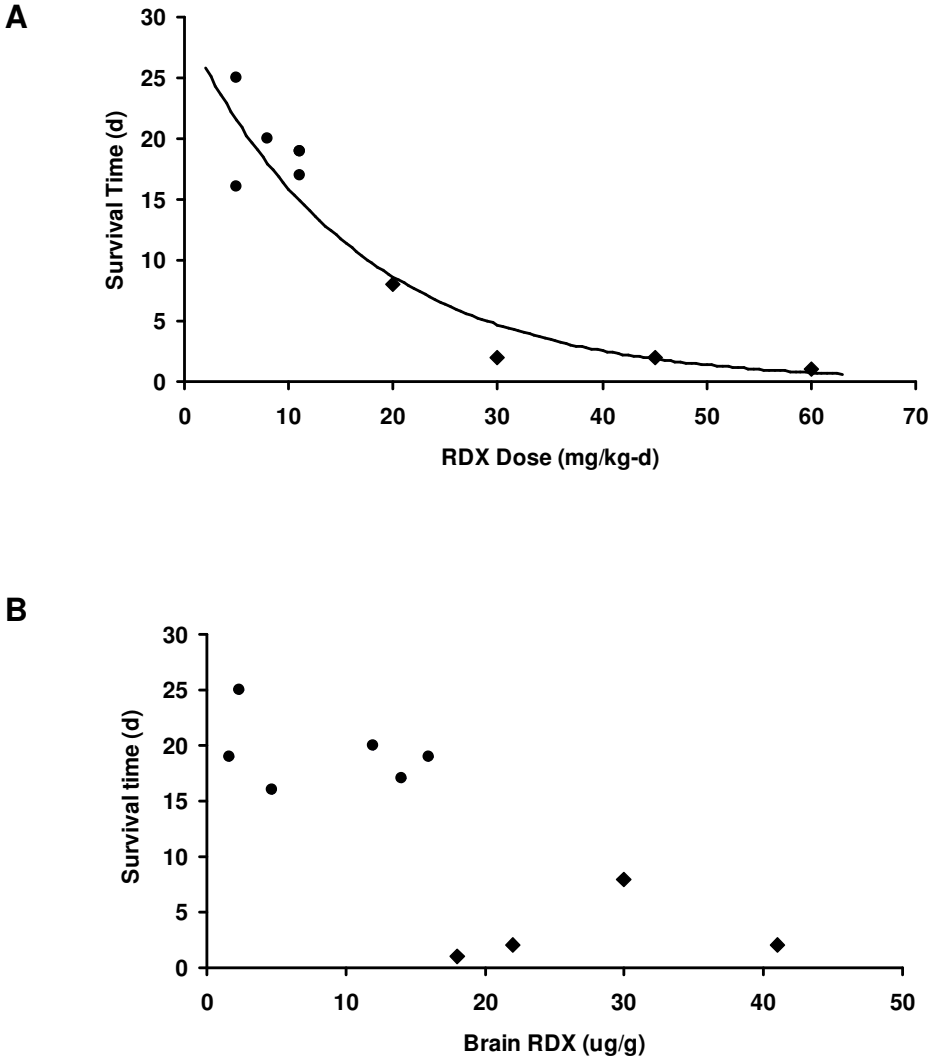

Figure 27. Changes in Survival Time Relative to Dose (A) and Brain RDX Concentration (B) in the Western Fence Lizard (*Sceloporus occidentalis)* Dosed with RDX for 60 Days (• = 60-d treatment group; ♦ = 14-d treatment group)

8. DISCUSSION.

a. Environmental exposure to military-unique compounds such as explosives and energetics have occurred through manufacturing and use for decades; however, their potential for adverse effects to wildlife has only recently been a concern. Regulators require toxicity information that is useful for evaluating risk from exposure at many contaminated lands that are formally used (Formally Used Defense Sites or FUDS), are being transferred (Base Realignment and Closure or BRAC), are designated Superfund sites, or where releases have unintentionally occurred. Regulatory authorities include Federal and state entities that are tasked with ensuring the public health and the environment and/or may have exclusive responsibilities in protecting specific species by law. Currently, this includes more than 90 percent of the bird species, threatened and endangered species, and species of special regional or state status. Additionally, changes in the health of such sentinel species may provide insights into concerns regarding human exposures from these substances.

b. The science of risk assessment is predicated on cause-and-effect relationships. Therefore, useful toxicity data require controlled studies where only exposure to the compound in question is varied. Hence, toxic relationships are determined to be compound related. The study designs used in these studies were based upon this foundation. The toxicity data provided herein provide the means to evaluate these questions from environmental contamination data for the four main classes of vertebrate wildlife (mammals, birds, reptiles, and amphibians). Given the great effort required to collect these data, it is generally accepted that toxicity data for species can be used for other species with a vertebrate class (i.e., data for any reptile species could be used to infer toxic responses to a specific field reptilian species); however, it is improper to extrapolate toxicity data across classes (i.e., assume that birds would react as rats would from exposure to the same compound). This logic is based upon the general physiological differences that occur in species between classes (reference 5). Occasionally, physiological differences for species within a class are profound where smaller subdivisions must be made. Our data confirm that mammalian species with extended gastrointestinal structures (e.g., herbivores—hindgut fermenting and possibly ruminants) are more sensitive to oral exposures of HMX than other mammalian monogastric species (e.g., rats and mice). This trend appears to hold for other nonmammalian monogastric species. HMX largely is not absorbed in species with simple gastrointestinal structures and short retention times; whereas, species with expanded gut physiologies and retention times are much more sensitive. These species tend to be obligate herbivores. Although only one hindgut fermenting species was tested, it is likely that other mammalian species with expanded gut physiology (e.g., ruminants) may be more sensitive; however, other studies have indicated aversion to HMX contaminated food (Johnson et al. 2005) suggesting that risk to those species may be minimal through avoidance of HMX in the field.

c. Given differences in methods and demographics between studies, relative toxicity comparisons are often difficult to make. However, a qualitative evaluation of species and LOAELs and NOAELs can be useful. Table 18 provides a brief synopsis of the results from the various studies.

Table 18. Comparison of LOAELs and NOAELs in Various Species from Exposures to Explosive Compounds and Their Breakdown Products

	Amphibians (mg/kg)*	**Reptiles (mg/kg-d)**	**Birds (mg/kg-d)**	**Mammals (mg/kg-d)**
TNT	373/42	25/15	70/20	8/2
DNT (2,4/2,6)	140/345	25/15	15/5 40/10	1.5/0.2 7/nf
RDX	3600/>680	5/2.5	8/3	8/4
A-DNT	850/250	15/5	14/3	760/170
HMX	~5000/na	~5000/na	~5000/na	10/5

Legend:
* = indicates soil concentrations.

d. Data from subchronic mammalian exposures to A-DNT suggest that *P. leucopus* is relatively less sensitive than Northern bobwhite or Western fence lizards. However, it must be acknowledged that the data for *P. leucopus* are based on a feeding study design; whereas, the others were via daily oral bolus (gavage). The toxicokinetics of the gavage method are quite different from the feeding method; however, they tend to be more sensitive and precise than the quantification of exposure through changes in feed weight, assumed to be attributable to ingestion. Changes in feed weight can be affected by spillage, urinary or fecal excretion. Heterogeneous distribution of the test material in the feed can also contribute to dose variability. Daily dose (exposure) was calculated from changes in feed weight coupled with changes in individual body mass. Together, these attributes suggest that daily exposure estimates are likely to be more variable than those from gavage studies. However, since the results of our homogeneity tests of compound in feed showed good distribution of the test substance and were consistent; any variation in consumption and exposure most likely reflects a combination of feed avoidance with the different dynamics of continuous daily

exposure. Regardless, such comparisons need to be made with caution.

e. It is also noteworthy that the data from the salamanders are presented as a concentration in soil and, as such, are not equivalent to data from the other columns which are expressed as oral daily dose (mg compound/kg body weight per day). Given the salamander's fossorial life strategy, it was impossible to separate dermal from oral exposures in each microcosm. Moreover, given the unique and important role of the skin in absorption, the importance of soil exposures could not be neglected (reference 4), particularly given that oral gavage and feeding procedures in these species are not practical.

f. These and other leveraged results provided the means for many peer-reviewed manuscripts that were published or under review. This was a primary goal of this effort since many of these methods and toxicology results were conspicuously absent in the literature. Additionally, greater weight is given to data which has undergone the scrutiny of peer review and is a criterion for inclusion in the EPA Soil Screening Level effort in TRV derivation (reference 7). All references derived from the work presented herein are listed in Appendix B, and the reader is encouraged to reference these citations for more information.

9. FURTHER RESEARCH. The studies presented herein provided valuable insights into the development of new study designs involving the use of new wildlife laboratory models. Overall, these new methods and models were successful with few caveats. Increasing the number of treatments, precise gavage administration of exposure, and the compound-specific consideration of absorption, disposition, metabolism, and excretion in choosing models greatly helped in the use of these data for field situations. Specifically, using the rabbit, a hindgut fermenting species, in the HMX exposure study showed that these and similar species with longer gut retention times would enhance exposure and likely be sensitive species. The development of other wildlife models is still needed, especially for avian and mammalian species (e.g. ruminant/hindgut and songbird models, respectively). More research is also needed in the development of full mesocosm terrestrial amphibian models, where oral exposure could be completed and quantified. Other data are needed to determine the relative sensitivities between species, and data for many other energetic compounds are needed to perform risk assessment.

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APPENDIX A

REFERENCES

Literature Cited

1. EPA. 1994. Method 8330 – Nitroaromatics, nitramines, and nitrate esters by high performance liquid chromatography (HPLC). U.S. Environmental Protection Agency, Office of Solid Waste, Washington, DC.

2. Feder P.I., D.W. Hobson, C.T. Olson, R.L. Joiner, and M.C. Matthews. 1991. Stagewise, adaptive dose allocation for quantal response dose-response studies. *Neuroscience and Biobehavioral Reviews,* 15: 109-114.

3. Johnson, M.S., L.S. Franke, R. B. Lee, and S.D. Holladay. 1999. Bioaccumulation of 2,4,6-trinitrotoluene and polychlorinated biphenyls through two routes of exposure in a terrestrial amphibian: is the dermal route significant? Environ Toxicol Chem. 18(5): 873-878.

4. Johnson M.S., S.D. Holladay, K.S. Lippenholz, J.L. Jenkins, and W.C. McCain. 2000. Effects of 2,4,6-trinitrotoluene in a holistic environmental exposure regime on a terrestrial salamander, *Ambystoma tigrinum*. *Toxicol Pathol* 28:334-341.

5. USACHPPM Technical Guide 254. 2000. Standard Practice for Wildlife Toxicity Reference Values. U.S. Army Center for Health Promotion and Preventive Medicine, Aberdeen Proving Ground, Maryland.

6. Johnson, M.S., R.M. Gogal, Jr., and C.T. Larsen. 2005. Food Avoidance Behavior to Dietary Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) Exposure in the Northern Bobwhite (*Colinus virginianus*). *Journal of Toxicology and Environmental Health* 68: 1349-1357.

7. EPA. 2000. Ecological Soil Screening Level Guidance- Interim Final. Eco-SSL Standard Operating Procedure #5: Wildlife TRV Data Evaluation. Attachment 4-4. U.S. Environmental Protection Agency, Region VIII, Denver, Colorado.

Other Publications

Beyer N.W., E.E. Connor, and S. Gerould. 1994. Estimates of Soil Ingestion by Wildlife. *J Wildl Manage,* 58(2): 375-382.

Campbell K.R. and T.S. Campbell. 2002. A logical starting point for developing priorities for lizard and snake ecotoxicology: a review of available data. *Environmental Toxicology and Chemistry* 21(5): 894-898.

Gibbons J.W., D.E. Scott, T.H. Ryan, K.A. Buhlmann, T.D. Tuberville, B.S. Metts, J.L. Greene, T. Mills, Y. Leiden, S. Poppy, and C.T. Winne. 2000. The global decline of reptiles, déjà vu amphibians. *Bioscience* 50: 653-666.

Hopkins W.A. 2000. Reptile toxicology: Challenges and opportunities on the last frontier in vertebrate ecotoxicology. *Environmental Toxicology and Chemistry* 19(10): 2391-2393.

Johnson M.S., M.W. Michie, M.A. Bazar, and C.J. Salice. 2005. Responses of oral 2,4,6-trinitrotoluene exposure to the common pigeon (*Columba livia*): a phylogenic and methodological comparison. *Int J Toxicol* 24:221-229.

Lambert M.R.K. 1997. Effects of Pesticides on Amphibians and Reptiles in Sub-Saharan Africa. *Rev. Environ. Contam. Toxicol* 150: 31-73.

Talent L.G., J.N. Dumont, J.A. Bantle, D.M. Janz, and S.G. Talent SG. 2002. Evaluation of Western fence lizards (*Sceloporus occidentalis*) and Western fence lizards (*Sceloporus undulates*) as laboratory reptile models for toxicological investigations. *Environmental Toxicology and Chemistry* 21(5): 899-905.

APPENDIX B

LIST OF PEER-REVIEWED PUBLICATIONS/PRESENTATIONS

Section I
Peer-Reviewed Manuscripts

Bazar, M.A., M.J. Quinn, Jr., K. Mozzachio, P. Beal, and M.S. Johnson. Toxicological responses of red-backed salamanders (*Plethodon cinereus*) to soil exposures of 2-amino-4,6-dinitrotoluene. (*in prep*).

Bazar, M.A., M.J. Quinn, Jr, K. Mozzachio, and M.S. Johnson. 2008. Toxicological responses of red-backed salamanders (*Plethodon cinereus*) to subchronic soil exposures of 2,4,6-trinitrotoluene (TNT*). Environ Toxicol Chem* 27(6): 1393-1398.

Johnson, M.S., C.A. McFarland, M.A. Bazar, M.J. Quinn, Jr., E.M. LaFiandra, and L.G. Talent. 2009. Toxicity of octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) in three vertebrate species. *Arch Contam Toxicol* (*epub ahead of print*).

Johnson, M.S. and C.J. Salice. 2009. Toxicity of Energetic Compounds in Wildlife Species. pp. 157-176 in Ecotoxicity of Explosives (Sunahara, G.I., G. Lotufo, R.G. Kuperman, and J. Hawari eds.). CRC Press, Boca Raton, Florida.

Johnson, M.S., J.S. Suski, and M.A. Bazar. 2007. Toxicological responses of red-backed salamanders (*Plethodon cinereus*) to subchronic soil exposures of 2,4-dinitrotoluene. *Environmental Pollution* 147: 604-608.

Johnson, M.S., H.I. Paulus, C.J. Salice, R.C. Checkai, and M. Simini. 2004. Toxicological and histopathological response of the terrestrial salamander *Plethodon cinereus* to soil exposures of 1,3,5-trinitrohexahydro-1,3,5-triazine (RDX)*. Arch. Environ Contam Toxicol* 47(4):496-501.

McFarland, C.A., M.J. Quinn, Jr., E.M. LaFiandra, M.A. Bazar, L.G. Talent, and M.S. Johnson. Exposure and toxic effects of oral 2-amino-4,6-dinitrotoluene in the western fence lizard (*Sceloporus occidentalis*) (*in prep*).

McFarland, C.A., M.J. Quinn, Jr., M.A. Bazar, L.G. Talent, and M.S. Johnson. 2009. Toxic effects of oral hexahydro-1,3,5-trinitro-1,3,5-triazine in the western fence lizard (*Sceloporus occidentalis*). *Environ Toxicol Chem* 28(5): 1043-1050.

McFarland, C.A., M.J. Quinn, M.A. Bazar, A.K. Remick, L.G. Talent, and M.S. Johnson. 2008. Toxicity of oral 2,4,6-trinitrotoluene exposure in the western fence lizard (*Sceloporus occidentalis*). *Environ Toxicol Chem* 27(5): 1102-1111.

Quinn, M.J., Jr., C.A. McFarland, E.M. LaFiandra, M.A. Bazar, and M.S. Johnson. 2010. Acute, subacute, and subchronic exposure to 2A-DNT (2-amino-6-dinitrotoluene) in the northern bobwhite (*Colinus virginianus).* *Ecotoxicology* (in press).

Quinn, M.J., Jr., C. McFarland, M. Bazar, E. Perkins, K. Gust, and M.S. Johnson. 2009. Sublethal effects of subacute exposure to RDX (1,3,5-trinitro-1,3,5-triazine) in the northern bobwhite (*Colinus virginianus*). *Environ Toxicol Chem* 28: 1266-1270.

Quinn, M.J., Jr., C. McFarland, M. Bazar, E. Perkins, K. Gust, and M.S. Johnson. 2007. Effects of subchronic exposure to 2,6-dinitrotoluene (DNT) in the Northern Bobwhite (*Colinus virginianus*). *Environ Toxicol Chem* 26: 2202-2207.

Section II
Presentations

Bazar, M.A. 2009. Toxicological Response of Red-Backed Salamanders to Soil Exposures of Individual Explosive Compounds, Metals, and Mixtures. Toxicology and Risk Assessment Conference (platform), Cincinnati, Ohio, 29 April 2009.

Johnson, M.S. 2008. Ecological risk assessment: Recent advancements and approaches for estimating risks to wildlife. Approaches for Managing Contaminated Upland Soils, SERDP/ESTCP Meeting, Washington D.C., December 2008 (invited).

Johnson, M.S. 2005. Toxicity of Energetic Compounds to Wildlife: Recent Data from Laboratory Investigations. Ecotoxicity of Energetic Materials, 1st Satellite Technical Workshop of KTA 4-32, The Technical Cooperative Program., Washington. D.C., December 2005.

Johnson, M.S. 2003. Effects of Energetic Compounds on Wildlife Species. Green Armament Technology Workshop. Cambridge, Massachusetts. November 2003 (invited presentation).

Johnson, M.S. and C.J. Salice. 2003. Influence of study design on vertebrate toxicity studies for applications in ecological risk assessments (platform presentation).

Wildlife as receptors at hazardous waste sites. Society of Environmental Toxicology and Chemistry (SETAC) Annual Meeting, Austin, Texas. November 2003.

McFarland, C.A., M.J. Quinn, Jr., E.M. LaFiandra, M.A. Bazar, L.G. Talent, and M.S. Johnson. 2008. Western fence lizards (*Sceloporus occidentalis*) as a model to investigate the toxicity of 2-amino-4,6-dinitrotoluene. Presented at the Society of Environmental Toxicology and Chemistry (SETAC) Annual Meeting, 16-20 November 2008, Tampa, Florida.

McFarland, C.A., M.J. Quinn, Jr., M.A. Bazar, L.G. Talent, and M.S. Johnson. 2008. Toxicity of 2A-DNT (2-amino-4,6-dinitrotoluene) in Western Fence Lizards (*Sceloporus occidentalis*). Presented at the CHPPM Toxicology Review Board, 21 October 2008, Aberdeen Proving Ground, Maryland.

McFarland, C.A., M.J. Quinn, Jr., M.A. Bazar, L.G. Talent, and M.S. Johnson. 2008. Toxicity of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in Western Fence Lizards (*Sceloporus occidentalis*). Presented at the CHPPM Toxicology Review Board, 22 April 2008, Aberdeen Proving Ground, Maryland.

McFarland, C.A., M.J. Quinn, Jr., M.A. Bazar, L.G. Talent, and M.S. Johnson. 2007. Toxicity of RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in Western Fence Lizards (*Sceloporus occidentalis*). Presented at the Society of Environmental Toxicology and Chemistry (SETAC) Annual Meeting, 11-15 November 2007, Milwaukee, Wisconsin.

McFarland, C.A., M.A. Bazar, M.J. Quinn, Jr., L.G. Talent, and M.S. Johnson. 2006. Toxicological Responses of the Western Fence Lizard (*Sceloporus occidentalis*) to Oral Exposures of 2,4,6-Trinitrotoluene (TNT). Presented at the Society of Environmental Toxicology and Chemistry (SETAC) Annual Meeting, 5-9 November 2006, Montreal, Canada.

Quinn, M.J., Jr., C. McFarland, M. Bazar, E. Perkins, K. Gust, and M.S. Johnson. 2009. A Comparison of Avian Toxicities to Explosive Compounds. Toxicology and Risk Assessment Conference.

Quinn, M.J., Jr., C. McFarland, E.M. Lafiandra, M. Bazar, E. Perkins, K. Gust, and M.S. Johnson. 2007. A Review of RDX Toxicity in the Northern Bobwhite. Society of Environmental Toxicology and Chemistry.

Quinn, M.J., Jr., C. McFarland, M. Bazar, and M.S. Johnson. 2006. Toxicological effects of oral exposure to 2,6-DNT in the Northern Bobwhite (*Colinus virginianus*). Society of Environmental Toxicology and Chemistry.

Quinn, M.J., Jr., E.M. LaFiandra, C. McFarland, M. Bazar, E. Perkins, K. Gust, and M.S. Johnson. 2006. Comparative toxicity of 2,4- and 2,6-DNT in northern bobwhite quail (*Colinus virginianus*). Tri-Service Ecological Risk Assessment Workgroup.

Section III
Posters

Johnson, M.S., C.A. McFarland, M.J. Quinn, Jr., M.A. Bazar, L. Talent, A. Hawkins, R. Porter, and R.M. Gogal, Jr. 2007. Derivation of toxicity data for munition compounds to support toxicity reference value derivations for wildlife. Partners in Environmental Technology Technical Symposium, SERDP/ESTCP Meeting, Washington D.C., November 2007.

McFarland, C.A., M.J. Quinn, M.A. Bazar, L.G. Talent, and M.S. Johnson. 2006. Toxicological Responses of the Western Fence Lizard (*Sceloporus occidentalis*) to Oral Exposures of 2,4,6-Trinitrotoluene (TNT). Partners in Environmental Technology Technical Symposium, SERDP/ESTCP Meeting, Washington D.C., November 2006.

Quinn, M.J., C.A. McFarland, M.A. Bazar, E.J. Perkins, K.A. Gust, and M.S. Johnson, 2006. Toxicological Effects of Oral Exposure to 2,6-DNT in the Northern Bobwhite (*Colinus virginianus*). Partners in Environmental Technology Technical Symposium, SERDP/ESTCP Meeting, Washington D.C., November 2006.

Section IV
Reports

McFarland, C.A., M.J. Quinn, Jr., M.A. Bazar, and M.S. Johnson. 2009. Effects of Oral Exposure to RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine) in the Western fence lizard (*Sceloporus occidentalis*). Toxicology Study 87-XE-06ED-07. U.S. Army Center for Health Promotion and Preventive Medicine (USACHPPM), Aberdeen Proving Ground, Maryland.